REMARKS

In the Final Office Action dated April 29, 2008, Claims 1, 2, 4-7, 9-15 and 17-20 are pending. Claims 6 and 12 are withdrawn from consideration as directed to non-elected species. Therefore, Claims 1, 2, 4, 5, 7, 9-11, 13-15 and 17-20 are currently under examination on the merits. Claims 14, 15 and 17-20 are rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enabling support. Claim 15 is rejected under 35 U.S.C. §102(b) as allegedly anticipated by Falsen et al. (*Journal of Systematic Bacteriology*, 217-221, 1999). Claim 20 is rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. Claims 1, 4, 9, 10, 13, 14, 19 and 20 are objected to for certain informalities.

This Response addresses each of the Examiner's objections and rejections. Applicants therefore respectfully submit that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

Claims 14, 15 and 17-20 are rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enabling support. The Examiner acknowledges that the specification is enabling for a method of inhibiting urogenital pathogen colonization of the urogenital tract in women by administering a therapeutically effective amount of *Lactobacillus iners* and a pharmaceutically acceptable carrier, wherein the *Lactobacillus iners* is administered orally or vaginally. However, the Examiner contends that the specification does not provide enablement for a method of maintaining a healthy urogenital flora in females by administering a therapeutically effective amount of at least one *L. iners* by any route of administration as allegedly recited in claims 14, 19 and 20. The Examiner also contends that the specification does not provide enablement for a method of treatment of any infection in a subject as allegedly encompassed by claim 19.

In an effort to favorably advance prosecution, Applicants have amended Claim 14 to recite "wherein said *Lactobacillus iners* is administered orally or vaginally." Support for the amendment is found in the specification on page 9, lines 20-21.

Applicants further submit that since the filing date of present application, references in the art have confirmed that *Lactobacillus* can be administered to the vagina by the oral route. See, e.g., Antonio et al. ("Colonization of the rectum by Lactobacillus species and decreased risk of bacterial vaginosis." *J Infect Dis*. 2005 Aug 1;192(3):394-8. Epub 2005 Jun 28) (copy of the Abstract is enclosed as **Exhibit N**); Vasquez et al. "Oral administration of Lactobacillus and Bifidobacterium strains of intestinal and vaginal origin to healthy human females: re-isolation from feces and vagina." *Microbial Ecol. Health Dis* 2005, 17: 15-20; Nishijima et al. "Probiotics affects vaginal flora in pregnant women, suggesting the possibility of preventing preterm labor." *J Clin Gastroenterol.* 2005, 39: 447-8) **(Exhibit O)**.

Notably, Nishijima et al. stated that "oral administration of probiotics (*lactobacilli*) can restore vaginal flora in pregnant women. Our results strongly support Reid's theory" See last paragraph of Nishijima et al. (emphasis added). Applicants respectfully submit that the Nishijima et al. reference provides verification for a method of treating an infection, irrespective of the type of infection, and for maintaining a healthy urogenital flora. The reference also confirms the viability of application of the claimed method to pregnant female subjects.

Moreover, Applicants provide the following additional examples to demonstrate that *lactobacilli* can have an effect on a wide range of diseases.

For example, studies have shown that *lactobacilli* can modulate immunity via ingestion and the gastrointestinal tract, and have an impact at distant sites. The therapy employed in the presently claimed method has been demonstrated to reduce the duration of respiratory infections (de Vrese et al. "Effect of *Lactobacillus gasseri* PA 16/8, *Bifidobacterium*

longum SP 07/3, *B. bifidum* MF 20/5 on common cold episodes: a double blind, randomized, controlled trial." *Clin Nutr.* 2005 Aug; 24(4):481-91. Epub 2005 Apr 21) **(Exhibit A)**, urinary tract infections (Lee et al., "Probiotics prophylaxis in children with persistent primary vesicoureteral reflux. *Pediatr Nephrol.* 200 7 Sep; 22(9):1315-20. Epub 2007 May 26) **(Exhibit B)**, cancer (Naito et al., "Prevention of recurrence with epirubicin and *Lactobacillus casei* after transurethral resection of bladder cancer." *J Urol.* 2008 Feb;179(2):485-90) **(Exhibit C)**, as well as produce by-products that provide the opportunity to treat many conditions (Martin et al., "Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model." *Mol Syst Biol.* 2008;4:157. Epub 2008 Jan 15) **(Exhibit D)**.

Accordingly, Applicants respectfully submit that the specification provides sufficient description for one skilled in the art to practice the presently claimed method for treatment of various infections usign *L. iners*, without undue experimentation. References in the art have also confirmed the effects of *L. iners* as claimed in the present application. Indeed, *L. gasseri*, *G. vaginalis* and *L. iners* have been reported to be the most frequent bacterial species in the vagina (Nikolaitchouk et al., "The lower genital tract microbiota in relation to cytokine-, SLPI- and endotoxin levels: application of checkerboard DNA-DNA hybridization (CDH)." *APMIS.* 2008 Apr;116(4):263-77) **(Exhibit E)**. Furthermore, Applicants respectfully submit that the same dose of *lactobacilli* administered (e.g., by mouth) can have multiple effects including modulating immunity in the gut (Baroja et al. "Anti-inflammatory effects of probiotic yogurt in inflammatory bowel disease patients." *Clin Exp Immunol.* 2007 Sep; 149(3):470-9 . Epub 2007 Jun 22) **(Exhibit F)**, treating HIV/AIDS (Anukam et al. "Yogurt containing probiotic *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 helps resolve moderate diarrhea and increases CD4 count in HIV/AIDS patients." *J Clin Gastroenterol.* 2008 Mar; 42(3):239-43)

(**Exhibit G**); treating bacterial vaginosis (Anukam et al. "Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: randomized, double-blind, placebo controlled trial. *Microbes Infect.* 2006 May;8(6):1450-4. Epub 2006 Mar 29) (copy of the Abstract is enclosed as **Exhibit P**).

The Examiner asserts that the use of an antibiotic is designed to kill bacteria, which use would not be appropriate to treat a viral infection. However, this assertion is no longer entirely correct. There are examples in the literature of antibiotics being used in human subjects having viral infections, to prevent superinfection by bacteria. See, e.g., Thorburn et al. ("High incidence of pulmonary bacterial co-infection in children with severe respiratory syncytial virus (RSV) bronchiolitis." *Thorax.* 2006 Jul;61(7):611-5. Epub 2006 Mar 14) (**Exhibit H**). Exhibit H demonstrates that "up to 40% of children with severe RSV bronchiolitis requiring admission to the PICU were infected with bacteria in their lower airways and were at increased risk for bacterial pneumonia." See Abstract of Exhibit H. There are also examples of antibiotics being used to improve weight of livestock animals or in cancer and HIV/AIDS therapy (Xing et al. "Novel beta-lactam antibiotics derivatives: their new applications as gene reporters, antitumor prodrugs and enzyme inhibitors." *Mini Rev Med Chem.* 2008 May;8(5):455-71) (Abstract is enclosed as **Exhibit I**). Xing et al. state that "recently, these 'old' antibiotics and their relevant derivatives have also found new applications as gene reporters, anti-cancer prodrugs and enzyme inhibitors." See Exhibit I (emphasis added).

Applicants submit that evidence has also demonstrated that direct vaginal administration of *lactobacilli* for treatment of infection has been successful (Anukam et al. "Clinical study comparing probiotic *Lactobacillus* GR-1 and RC-14 with metronidazole vaginal gel to treat symptomatic bacterial vaginosis." *Microbes Infect.* 2006 Oct;8(12-13):2772-6. Epub

2006 Sep 11) (copy of the Abstract is enclosed as **Exhibit Q**). Given that *L. iners* is a frequent colonizer of the vagina and has attributes suitable for curing bacterial vaginosis (see the Saunders et al. reference, which was provided to the Examiner in previous response dated February 4, 2008), *L. iners* would be perfectly capable of treating infection.

The Examiner appears to be of the opinion that the healthy vaginal flora requires multiple species of *lactobacilli*. Contrary to the Examiner's opinion, Applicants respectfully submit that vaginal region is often only colonized by one dominant strain. See Heinemann and Reid, ("Vaginal microbial diversity among postmenopausal women with and without hormone replacement therapy." *Can J Microbiol*. 2005 Sep;51(9):777-81) (copy of the Abstract is enclosed as **Exhibit R**) and Fredricks et al. ("Molecular identification of bacteria associated with bacterial vaginosis. *N Engl J Med*. 2005 Nov 3;353(18):1899-911) **(Exhibit J)**.

The Examiner contends that there are different bacterial strains, which have different functions. In this respect, Applicants submit that although differences exist among strains of bacteria, there is a consistency that classifies one bacterium within a certain family of like strain. Thus, *lactobacilli* are not like *E. coli*. Applicants submit that the commonality that *L. iners* has with other *lactobacilli* strains, such as *L. rhamnosus* GR-1 and *L. reuteri* RC-14, is that all *lactobacilli* strains inhabit, colonize and improve the well-being of the vagina. The precise mechanisms involved in countering disease can differ, but the end result is the same – improved health of the urogenital tract.

The Examiner relies on Ferri et al. (*Clin. Microbio*. 1016-18, 2007) to question the protective role of *L. iners*. Applicants observe that Ferri et al. state that "*L. iners* is prevalent in grade Ib, a variant of normal, and in grade III, representing bacterial vaginosis (BV). We speculate that *L. iners* is a transitional species and that an L. crispatus-predominant species composition represents a stable flora." Applicants respectfully submit that since only the

inventors' group has actually performed experiments using *L. iners*, the Ferris et al. reference is the first to evaluate *L. iners* in light of its probiotic potential. However, Applicants respectfully submit that Ferri et al. represented only one view of the role of *L. iners* in health at an earlier time. A recent study shows that *Lactobacillus iners* was the predominant *Lactobacillus* found in over 50% samples and this was associated with a healthy vagina. See Dahn et al. ("Effect of bacterial vaginosis, *Lactobacillus* and Premarin estrogen replacement therapy on vaginal gene expression changes." *Microbes Infect.* 2008 May;10(6):620-7. Epub 2008 Feb 21) **(Exhibit K)**. Dahn et al. recognize that *L. iners* was not associated with BV, as previouly suggested in the art.

Therefore, Applicants respectfully submit that in view of the specification and well known knowledge in the art, one skilled in the art can make and use the appropriate *lactobacilli* in the methods as claimed in the present application, without undue experimentation. In view of the foregoing argument and the amendment to the claims, the rejection of Claims 14, 15 and 17-20 under 35 U.S.C. §112, first paragraph, as allegedly lacking enabling support, is overcome and withdrawal thereof is respectfully requested.

Claim 15 is rejected under 35 U.S.C. §102(b) as allegedly anticipated by Falsen et al. (*Journal of Systematic Bacteriology*, 217-221, 1999). The Examiner alleges that Falsen et al., teaches a new isolated species of *Lactobacillus*: *L. iners* that grows in an agar culture supplemented with 5% horse blood at 37°C in air plus CO_2. According to the Examiner, a prebiotic broadly encompasses <u>any growth media</u> that enhances the growth of the bacteria (e.g., serum added to the media). Therefore, the Examiner concludes that the Falsen et al. anticipate the present invention by teaching a new isolated species of *Lactobacillus: L. iners* that grows in an agar culture supplemented with 5% horse blood at 37°C in air plus CO_2, which is further prepared in SDS (Sodium Dodecyl Sulfate) for protein quantification.

Applicants observe that, as acknowledged by the Examiner, Falsen et al. simply identify a single *L. iners* organism, which identification by itself, without more, does not teach or suggest that the *L. iners* organism can be placed in a pharmaceutical or food carrier and delivered for therapeutic purposes. Applicants respectfully submit that the agar media used by Falsen et al. is well known to be capable of growing many types of microorganisms including pathogenic ones. In this regard, Applicants respectfully submit that contrary to the Examiner's allegation that a prebiotic broadly encompasses any growth media that enhances the growth of the bacteria, a prebiotic recited by Claim 15 is clearly defined by the present application as "a . . . substrate that . . . selectively enhances the growth and/or the metabolic activity of a bacterium or a group of bactria. A prebiotic also includes a nutrient utilized by lactobacilli or bifidobacteria to stimulate and/or enhance growth of lactobacilli or bifidobacteria relative to pathogenic bacteria." See page 7, lines 5-9 of the specification (emphasis added). Thus, Falsen et al. do not teach or suggest that blood agar can be a prebiotic. Indeed, Applicants respectfully submit that by definition, blood agar is not a prebiotic because it cannot selectively enhances the growth of a bacterium or a group of bactria relative to pathogenic bacteria. Moreover, blood agar cannot possibly function as a prebiotic because its contents can be digested in the body and do not require *lactobacilli*. Blood agar is not a carrier either. In fact, many organisms, including pathogens, can digest this agar and serum, making blood agar a very poor choice for a carrier. Therefore, one skilled in the art in view of the present application will recognize that blood agar and serum are not prebiotics or suitable carrier vehicles for *lactobacilli* application as claimed.

Applicants submit additional evidence to demonstrate that blood is not a prebiotic (the definition of which can be found in "Food component that confer a health benefit on the host through modulation of the microbiota" - *Food Quality and Standards Service (AGNS) Food and Agriculture Organization of the United Nations (FAO)* September 15-16, 2007) (**Exhibit L**).

As such, Applicants respectfully submit that the Falsen et al. reference does not teach or suggest each and every element recited in Claim 15. Therefore, the rejection of Claim 15 under 35 U.S.C. §102(b) as allegedly anticipated by Falsen et al. is overcome and withdrawn thereof is respectfully requested.

Claim 20 is rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. The Examiner essentially contends that the phrase "displacing vaginal pathogens" as recited in Claim 20 is not described in the specification. The Examiner alleges that Claim 20 is directed to both indigenous and endogenous vaginal pathogens.

The Examiner asserts that the recited vaginal pathogen encompasses exogenous microorganisms, such as HIV and herpes. To this end, Applicants respectfully submit that the present invention is partly predicated upon the recognition that *L. iners*' ability to restore and maintain health of the urogenital tract begins with its ability to reduce the infectious agents which originate in the intestine and ascend from the rectum to the vagina. Neither HIV nor herpes simplex virus infects in this manner.

The present invention recognizes that bacterial vaginosis organisms and the condition itself were displaced coinciding with the presence of *L. iners*. Since bacterial vaginosis has a prevalence of 29% (Allsworth and Peipert, "Prevalence of bacterial vaginosis: 2001-2004 National Health and Nutrition Examination Survey data." *Obstet Gynecol.* 2007 Jan;109(1):114-20) (**Exhibit M**), the recognition of *L. iners* in healthy subjects by the present invention proves that *L. iners* has anti-infective properties. Thus, Applicants submit that the present inventors had possession of the subject matter at the time of the present application was filed.

In an effort to favorably advance prosecution, Applicants have also amended Claim 20 to recite "wherein the vaginal pathogens are gastrointestinal or urogenital bacterial flora."

Support for the amendment is found on bottom of page 7 of the specification. No new matter is introduced.

In view of the above discussion and amendment to Claim 20, the rejection of Claim 20 under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement is overcome and withdrawal thereof is respectfully requested.

The Examiner objects to the amendments to the claims. The Examiner alleges that the amendment filed on December 18, 2007 does not comply with the requirements of 37 C.F.R. §1.121(c). The Examiner alleges that changes in the text of currently amended withdrawn claim 13 filed on February 4, 2008 was not completely marked with respect to the previously presented claim 17, filed on July 26, 2007. Specifically, currently amended claim 17 has been amended, thus it is not identified with the proper status in the claim listing.

Applicants respectfully submit that no response was filed on December 18, 2007. Claim amendments that were filed on February 4, 2008 and July 26, 2007 both fullycomplied with the requirements of 37 C.F.R. §1.121(c). Applicants respectfully request clarification of this objection from the Examiner.

Claims 1, 9, 13, 14, 19 and 20 are objected to for certain informalities. The Examiner states that Claims 1, 9, 13, 14, 19 and 20 recite the phrase "of at least one *Lactobacillus iners*" which could be interpreted as administering solely one bacterium. The Examiner suggests that the deletion of the phrase "of at least" will help to clarify the meaning of the claims. Applicants have amended the claims in accordance with the Examiner's suggestion.

Claims 4 and 10 are objected to for certain informalities. The Examiner states that Claims 4 and 10 recite the phrase "further comprising a second probiotic. " According to the Examiner, claims 4 and 10 depend on claims 1 and 9, respectively. The Examiner asserts that one of skilled in the art understand that the second probiotic is administered to the methods of

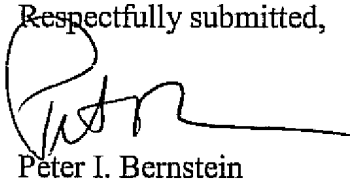
claims 4 and 10. As such, the Examiner suggests that the insertion of the phrase "comprising administering" will help to clarify the meaning of the claims. Applicants have amended the claims in accordance with the Examiner's suggestion.

In addition, the Examiner states that Claims 4 and 10 recite the phrase "a second probiotic". Claims 4 and 10 depend on claims 1 and 9, respectively. However, the Examiner indicates that claims 1 and 9 do not explicitly recite administering a first probiotic *Lactobacillus iners*. The Examiner suggests that the insertion of the phrase "a first probiotic" will help to clarify the meaning of the claims. Applicants have amended the claims in accordance with the Examiner's suggestion.

Therefore, the objections of Claims 1, 4, 9, 10, 13, 14, 19 and 20 are obviated and withdrawal thereof is respectfully requested.

In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,



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Encs: Exhibits A-R

EXHIBIT A

Clinical Nutrition (2005) 24, 481–491

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ORIGINAL ARTICLE

Effect of *Lactobacillus gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3, *B. bifidum* MF 20/5 on common cold episodes: A double blind, randomized, controlled trial

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KEYWORDS
Probiotics;
Common cold;
Respiratory tract infections;
Immune system

Summary
Background & aims: The aim of this study was to investigate whether the consumption of *Lactobacillus gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3, *B. bifidum* MF 20/5 (5×10^7 cfu/tablet) during at least 3 months influences the severity of symptoms and the incidence and duration of the common cold.
Methods: A randomized, double-blind, placebo-controlled intervention study was performed over at least 3 months during two winter/spring periods. Four hundred and seventy nine healthy adults (aged 18–67) were supplemented daily with vitamins and minerals with or without the probiotic bacteria. Cellular immune parameters were evaluated in a randomly drawn subgroup of 122 volunteers before and after 14 days of supplementation. During common cold episodes, the participants recorded symptoms daily. Stool samples were collected before and after 14 days of probiotic supplementation to quantify fecal Lactobacilli and Bifidobacteria using qRT-PCR.

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Results: The total symptom score, the duration of common cold episodes, and days with fever during an episode were lower in the probiotic-treated group than in the control group: 79.3±7.4 vs. 102.5±12.2 points ($P = 0.056$), 7.0±0.5 vs. 8.9±1.0 days ($P = 0.045$), 0.24±0.1 vs. 1.0±0.3 days ($P = 0.017$). A significantly higher enhancement of cytotoxic plus T suppressor cells (CD8+) and a higher enhancement of T helper cells (CD4+) was observed in the probiotic-treated group. Fecal lactobacilli and bifidobacteria increased significantly after probiotic supplementation.

Conclusions: The intake of probiotic bacteria during at least 3 months significantly shortened common cold episodes by almost 2 days and reduced the severity of symptoms.

Introduction

Despite great advances in medicine, common virus infections such as the common cold or influenza continue to cause a considerable economic burden, due to loss in productivity and high medical costs.[1]

Several strains of probiotic microorganisms have a positive influence on a broad range of functions related to the host's defense system.[2,3] However, whereas many publications describe effects of probiotic microorganisms on intestinal pathogens,[4] there are few studies investigating their effect on infections in other organ systems, especially the respiratory tract.[5]

The first evidence that probiotic strains could be effective in respiratory tract infections came from mice, where the oral administration of *Bifidobacterium breve* YIT4064 augmented anti-influenza IgG production and protected against influenza infection.[6] A study on 129 children with acute respiratory tract infections showed that the administration of a bifidobacteria preparation stimulated T-cell and B-cell immunity, natural killer cells and interferon release.[7] Finnish children in day care centers who consumed milk containing *Lactobacillus rhamnosus* GG (ATCC 53103) during seven months in winter had 17% fewer respiratory tract infections than the control group.[8] The administration of an *Enterococcus faecalis* preparation (Symbioflor®) resulted in fewer relapses of chronic, recurrent, respiratory tract infections, especially bronchitis and sinusitis.[9,10] In a 3 weeks observation period in elderly people, the duration of gastrointestinal and respiratory illnesses was significantly lower in the group receiving *L. casei* DN-114001 than in the control group.[11] These studies, however, do not distinguish between bacterially and virally induced infections. In two of them gastrointestinal infections were considered together with respiratory infections in the primary parameter.

The aim of the present study was to investigate whether the consumption of certain probiotic bacteria during a period of at least 3 months in winter/spring affects the severity of symptoms, the incidence and duration of naturally acquired common cold infections, and the cellular immune response in otherwise healthy adults.

The dietary supplement used in the present study was thought to be an "all-in-one" product, which was designed to secure adequate vitamin and mineral intake according to the dietary allowances. Adequate supply was shown to have impact on immunity. Probiotics were added with the aim of enhancing immune defense. Since we wanted to investigate the probiotic effect, the control group was supplied with the same amount of vitamins and minerals as the probiotic-treated group.

Methods

Study design and ethics

The study was carried out double-blind, randomized, and placebo-controlled, with two parallel arms. It was approved by the ethics committee of the Medical Faculty of the Christian-Albrechts-University Kiel, Germany. All participants gave their written informed consent before inclusion in the study.

Participants

Volunteers were recruited by advertisements. A total of 479 healthy women and men were enclosed after physical examination. Four hundred and fifty-four of them completed the study. The exclusion criteria were: laboratory parameters outside the normal range, known congenital or acquired immune defects, allergies and other chronic or acute

diseases requiring treatment, alcohol or drug misuse or both, pregnancy or lactation, interfering dietary habits, or vaccination against influenza within the last 12 months.

During the study, neither immune stimulating medication nor abnormal physical exercise was allowed. The volunteers were asked to maintain their usual eating habits but to refrain from eating products containing prebiotics and/or probiotics according to labeling and the following fermented milk products: yogurts, kefir, crème fraiche, sour crème, and sour milk. Cheese was allowed for compliance reasons. Curd cheese and butter milk, which are fermented using mesophilic starter cultures, which do not include probiotic strains, were also permitted. The participants were briefed in an informative meeting and in written form before the study started.

Test preparations, blinding, and randomization

Test preparations were given in tablets with the same appearance, smell and taste. They contained either 5×10^7 cfu of the spray dried probiotic bacteria with vitamins and minerals (hereafter called probiotics) or just the vitamin mineral preparation (hereafter called control). The vitamins and minerals in this preparation were according the EC recommended daily allowance (RDA) as far as existing (for details see Table 1).

The probiotic strains used in this study were *L. gasseri* PA 16/8, *B. longum* SP 07/3, *B. bifidum* MF 20/5 (*Tribion harmonis*™). The number of viable probiotic bacteria declared for the product during the study period was guaranteed by long-term stability tests carried out by the supplier. These bacterial strains have been shown to help protect mice from intestinal infections with pathogenic *Escherichia coli* 0-136.[12]

All test preparations were prepared, packaged, and randomized by the supplier (Merck, Consumer Health Care) who labeled the packages with identification numbers in order to fulfill the criteria of a double-blind trial.

The volunteers were assigned to these identification numbers, according to recruitment.

Intervention

Two hundred and forty-four participants were observed during a 3 month period (between January and May 2001), and 237 participants during a 5.5 month period (between December 2001 and June 2002). The volunteers were asked to take one tablet of the test preparation per day, in addition to their usual diet. They collected the tablets from the Federal Research Centre for Food and Nutrition every 4 weeks. This ensured close contact to the volunteers in order to ascertain optimal compliance.

Cellular immune response was assessed by flow cytometry in a randomly drawn subgroup of 122 participants (61 per study group) before and after 14 days of supplementation in the first study period. As a randomized block design was used, we were ensured that the groups were of the same size.

During respiratory tract infection episodes, the participants used questionnaires to daily record the symptoms mentioned. An episode was defined as the appearance of at least one specific respiratory tract symptom (nasal, pharyngeal or bronchial). With the aim of excluding bacterial respiratory tract infections, episodes were not considered if a participant received antibiotic treatment. On day two of each episode, nasal secretions were collected for virus identification.

Outcome measures

The following parameters were calculated:

- single specific symptoms that appeared during common cold episodes,
- total symptoms score expressing overall severity of each episode (primary parameter),
- the duration and incidence of common cold episodes,
- the cellular immune response,
- the type of viruses identified, and
- fecal lactobacilli and bifidobacteria.

Clinical assessment of common cold episodes

The questionnaires for assessing the common cold episodes were based on studies by Stansfield et al. and Mossad et al.[13,14] Volunteers noted nasal symptoms (running nose, stuffed nose, blowing the nose, yellow secretion, bloody secretion, sneezing), pharyngeal symptoms (scratchy throat, sore throat, hoarseness), and bronchial symptoms (cough, secretion, yellow secretion), headache, myalgia, conjunctivitis (reddish eyes), fatigue, loss of appetite, and fever (oral temperature >37.7 °C) daily. Severity of nasal, pharyngeal, and bronchitis symptoms, as well as headache, myalgia, and conjunctivitis was each graded as: no symptoms = 0, mild symptoms = 2, moderate symptoms = 4, and severe symptoms = 6. Sneezing, fatigue, and loss of appetite were graded as: yes = 1, no = 0. Fever was

Table 1 Composition of the test preparations.

	Probiotics plus vitamins and minerals	Control (vitamins and minerals)
	Per tablet	
Probiotic bacteria (cfu):	5×10^7	
(*Lactobacillus gasseri* PA 16/8,	4×10^7	—
Bifidobacterium longum SP 07/3,	5×10^6	
B. bifidum MF 20/5)*	5×10^6	
Vitamin C (mg)	60	60
Vitamin E (mg)	10	10
Nicotinamide (mg)	18	18
Vitamin A (µg)	800	800
Vitamin K1 (µg)	30	30
Vitamin B6 (mg)	2	2
Vitamin B1 (mg)	1.4	1.4
Vitamin B2 (mg)	1.6	1.6
Vitamin D (µg)	5	5
Vitamin B12 (µg)	1	1
Folic acid (µg)	200	200
Biotin (µg)	150	150
Pantothenic acid (mg)	6	6
Calcium (mg)	40	40
Phosphorus (mg)	16	16
Potassium (mg)	5	5
Chloride (mg)	4.5	4.5
Magnesium (mg)	5	5
Iron (mg)	14	14
Zinc (mg)	15	15
Manganese (mg)	2	2
Selenium (µg)	30	30
Silicon (µg)	2	2
Chromium (µg)	25	25
Molybdenum (µg)	25	25
Iodine (µg)	150	150

Ingredients: Microcrystalline cellulose, Calcium ascorbate granulate, Lactose monohydrate, Tricalcium phosphate anhydrous. *Coating*: (Shellac, Hydroxypropyl methyl cellulose, Acetylated monoglycerides povidone glycerol), Iron (II) sulphate hydrate, Vitamin E preparation (DL-Alpha tocopheryl acetate), Glucose, Selenium yeast, Probiotic bacteria powder, Glycerol monostearate, Nicotinamide, Zinc oxide, Sodium carboxymethyl cellulose, Povidone, Potassium chloride, Magnesium oxide, Calcium pantothenate, Crospovidone, Vitamin A preparation, Manganese (II) sulphate monohydrate, Magnesium stearate, Iron oxide, Vitamin K1 glucose trituration, Vitamin D3 powder, Pyridoxine hydrochloride, Thiamine mononitrate, Riboflavin, Colloidal silicon dioxide, Cyanocobalamine glucose trituration, Folic acid, Potassium iodide, Biotin, Chromium (III) chloride hexahydrate, Sodium molybdate dihydrate.
**Tribion harmonis*™.

always scored as 6. These symptom scores were combined to produce a daily symptom score with a maximum of 45 per day. The daily recorded scores were added up over the illness period to produce a total symptom score.

Flow cytometry

Blood cells were analyzed with the aid of a EPICS® XL-MCL flow cytometer (Beckman Coulter®, Krefeld, Germany). Lymphocytes (CD45+), B-lymphocytes (CD45+, CD19+), T-lymphocytes (CD45+, CD3+), T_H cells (CD45+, CD3+, CD4+), T_S plus T_C cells (CD45+, CD3+, CD8+), and natural killer cells (CD 45+, CD 56+) were differentiated using a Beckman Coulter tetraChrome test®. Cell populations were calculated by Coulter® System II Software, Version 3.0, and Coulter® tetraONE SYSTEMsoftware. In order to determine T-lymphocyte activation, whole blood was incubated with 5 µg PHA/ml or 20 µg PHA/ml for 2 h (the control was the same treatment with PBS). CD69

expression was chosen as an early activation marker.[15] The cells were stained with CD3, CD4, CD8, and CD69 monoclonal antibodies (IO Test® Coulter Immunotech). The number of granulocytes and monocytes was determinated using forward and sideward scatter. Phagocytic activity was investigated by the Orpegen PhagoTest®. This test kit determinates phagocytic function of granulocytes and monocytes in whole blood by measuring ingestion of fluorescein-labeled *E. coli*.[16]

Virus identification

For virus identification samples of nasal discharge were used. Knowing that this material warrants lower sensitivity than nasopharyngeal aspirate, we chose these samples for compliance reasons since aspiration is associated with some discomfort. The volunteers blew their noses into a cellulose wipe (Kimwipes® Lite 100© Kimberly-Clark Corporation). The samples were refrigerated and stored in lockable plastic containers at −20 °C until use. Viruses were identified after the extraction of nucleic acid (QIAamp® Viral RNA Mini Kit, Qiagen, Hilden, Germany) by means of RT-PCR or PCR. Reverse transcription and the first PCR for the detection of enterovirus, respiratory synzitial virus (RSV) and parainfluenza 3 virus RNA was performed in a one step reaction, using the cMasterRTplusPCR system (Eppendorf, Hamburg). Primers were supplied by Tib Molbiol (Berlin). In order to enhance the specificity, PCR products were used as templates in a second PCR round (nested PCR), using the Hot Master Taq polymerase (Eppendorf) in the corresponding buffer system. PCR products were analyzed by gel electrophoresis on 2% E-Gels (Invitrogen). The presence of parainfluenza-1, 2, and 4, metapneumo- A and B, influenza- A and B, and adenovirus, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae* was also sought.

PCR products from enterovirus RT-PCR were further characterized by cycle sequencing (performed by Sequa, Gutting). The obtained sequence data were analyzed using the software package Lasergene (DNA Star) and were identified by BLAST search.

Quantification of fecal lactobacilli and bifidobacteria

In order to assess the increase of fecal count of the bacteria administered, we collected stool samples of 20 volunteers (10 women and men each) from the study cohort in a separate trial. Before the first stool samples were collected, the volunteers were asked to refrain from eating the products mentioned above for 14 days. Thereafter, the volunteers took the dietary supplement containing the probiotic bacteria daily for 14 days still refraining from eating the mentioned edibles until stool samples were collected the second time. Samples were frozen in liquid nitrogen and stored at −80 °C until use.

DNA of the stool samples was isolated with the FastDNA SPIN Kit for Soil (BIO 101, Calsbad, USA) after mechanical homogenization (FastPrepTM FP 120 instrument, Bio 101, Calsbad USA) according to the manufacturer's instructions. The DNA was checked by 1.5% agarose gel electrophoresis. DNA concentrations were determined using PicoGreen™ (dsDNA Quantification Kit, Molecular Probes, Leiden, The Netherlands).

Quantitative real time PCR assays were performed and optimized to detect the concentration of *Bifidobacterium* spp. and *Lactobacillus* spp. in the stool samples. Primers and probes of the real time assays are based on the 16S rRNA gene (Table 2). All primers and probes used in this study hybridize to variable regions of the 16S rRNA gene specific for the bifidobacteria and lactobacillus group. The primer pair LAC_1_RT and LAC_2_RT produce a 16S rDNA fragment of ~320 bp length and

Table 2 Characteristics of primers and probes used for the real time PCR assays.

Primer/Probe	Position*	Direction	Dye	Sequence (5′–3′)	T_m (°C)	Reference
LAC_1_RT	350-370	Forward	—	GCAGCAGTAGGGAATCTTCCA	58.0	Modified from Walter et al.[17]
LAC_2_RT	677-690	Reverse	—	GCATTTCACCGCTACACATG	60.2	Modified from Walter et al.[17]
LACTO	455-471	—	FAM†	AGGCCAGTTACTACCT	66.0	Ott et al.[18]
Bif_164	164-181	Forward	—	GGGTGGTAATGCCGGATG	59.3	Langendijk et al.[19]
Bif_662	662-679	Reverse	—	TTCCACCGTTACACCGGGAA	62.0	Langendijk et al.[19]
Bifido_584	584-601	—	FAM†	TGAAAGTCCATCGGCTTA	65.6	Modified from Bernhard et al.[20]

*According to the *Escherichia coli* reference numbering.
†FAM = 6-carboxyfluorescin.

includes bacterial species of the following groups: *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella*.[17] The probe LACTO is a minor groove binder (MGB) probe designed using the PROBE_Design tool of the ARB software package (ARB sofware package, www.arb-home.de).[18] The probes are labeled with the fluorescence dye FAM (6-carboxyfluorescin). The specificity of the LACTO probe encloses most species of the lactobacillus group, among them *L. acidophilus*, *L. delbrueckii*, *L. johnsonii*, *L. gasseri*, and *L. casei*. For optimization of the assay and generation of standard curves, serial dilutions of *L. acidophilus* and *L. gasseri* strains were used,[18] the latter cultivated from the probiotic compound. The primer pair Bif_164 and Bif_662 produces a fragment of ~500 bp length. The probe Bifido_584 is a FAM-labeled minor groove binder (MGB) probe modified from Bernhard and Field.[20] Both the primers and the probe hybridize to bifidobacterium specific regions of the 16S rRNA gene, including *B. longum*, *B. bifidum*, *B. adolescentis*, and *B. infantis*.[19,20] For optimization of the assay and generation of standard curves, serial dilutions of *B. adolescentis*, *B. longum*, and *B. bifidum* strains were used. The *B. bifidum* strain was cultivated from the probiotic compound. Real time PCR was performed with the aid of an ABI PRISM™ 7700 Sequence Detector using TaqMan™ Universal PCR 2x Master Mix (Applied Biosystems, Foster City, CA, USA), primer (0.4 µM), probe (0.2 µM), and 100 ng of sample DNA in a final volume of 50 µl per reaction with an initial hold of 50 °C for 2 min to activate No Amp Erase™ UNG, a hold of 95 °C for 10 min to activate AmpliTaq™ Gold Polymerase followed by 50 cycles for 30 s at 95 °C, 60 °C for 1 min, and 72 °C for 2 min. The PCR products were checked for size and side bands by gel electrophoresis using 1.5% agarose gels. The real time PCR results were averaged from two independent experiments and related to 1 g of stool.

Statistical power and analyses

Information on the duration of common cold episodes was obtained a year before from interviews with residents of Kiel and the surrounding area. A minimum of 61 participants per group was calculated from: a mean duration of 6.0 (SD 1.7) days, $\alpha = 0.05$, power $(1 - \beta) = 0.1$ and an expected reduction of 1 day in the duration of episodes in the probiotic-treated group, according to the results of a study on oseltamivir.[21] Assuming that only 60% of the participants would catch a cold and allowing for 20 withdrawals, a total of 120 participants was to be recruited per group and intervention period.

The minimum of 61 participants was in accordance with a study by Hoheisel et al., in which 60 volunteers with initial cold symptoms were necessary per group in order to demonstrate the significantly positive effect of an immune stimulating *Echinacea purpurea* extract on the duration of common cold episodes.[22]

The sample size calculation was based on an entire common cold season from autumn to spring. For technical reasons the study did not start before January 2001. Therefore, we decided to extend the study to a second period. In order to avoid bias new volunteers were recruited in the second period, who were randomized separately securing equal numbers in the probiotic-treated group and control group in each period.

Data are presented as mean ± standard error of the mean (SEM). Differences between the probiotic-treated group and the control group were analyzed by independent Student's *t*-tests for the duration of common cold episodes, days with fever, and differences in cell counts between day 14 and day 0. The incidence of infections was compared between the two groups using the χ^2 test. The differences in symptom scores were assessed by the non-parametric Mann-Whitney test. As far as the available literature shows that probiotic bacteria, if they have any effect at all, stimulate immune response, one-tailed tests were chosen. Bacterial counts in stool samples were compared by sign test. A value of $P \leqslant 0.05$ was regarded as statistically significant. All statistical analyses were performed using the software package Statgraphics Plus®, version 4.1 (Manugistics, Rockville, USA).

Results

Participants

Two hundred and forty-two participants started in the study period between January and May 2001: 121 in the probiotic-treated and 121 in the control group. There were 5 withdrawals in the probiotic-treated and 3 in the control group. Two hundred and thirty-seven participants started the study period between December 2001 and June 2002: 117 in the probiotic-treated and 120 in the control group. There were 8 withdrawals in the probiotic-treated and 9 in the control group. In the first study period one withdrawal was due to pregnancy, the reminding were due to non-compliance. There was no report of adverse events. The distribution of sex and age was equal in the verum and the control group (Table 3). Altogether data from the 454

Table 3 Age and sex of volunteers.

		Probiotics+vitamins and minerals	Control (vitamins and minerals)	Total
Age				
Period I		36±12	36±13	36±13
Period II		39±12	41±14	40±13
Total		37±12	38±14	38±13
Sex				
Period I	W	75	73	148
	M	46	48	94
	W+M	121	121	242
Period II	W	77	69	146
	M	40	51	91
	W+M	117	120	237
Total	W	152	142	294
	M	86	99	185
	W+M	238	241	479

W, women; M, men.
Period I lasted from January 2001 to May 2001.
Period II lasted from December 2001 to June 2002.

Table 4 Effect of probiotic bacteria (5×10^7 cfu/day) on symptoms of the common cold episodes with more than one symptom in detail during the observation period.

	Probiotics+vitamins and minerals	Control (vitamins and minerals)	*P*
Duration (days)	7.0±0.5	8.9±1.0	0.045*
Symptom scores (points)			
Nasal symptoms (inclusive sneezing)	29.5±2.7	36.8±3.7	0.053†
Pharyngeal symptoms	12.9±1.7	17.1±2.5	0.051†
Bronchial symptoms	13.2±2.1	19.1±2.7	0.011†
Headache	8.5±1.2	10.0±1.6	0.245†
Myalgia	5.3±1.2	5.5±1.4	0.142†
Conjunctivitis	3.5±0.9	2.8±1.0	0.925†
Fatigue	2.7±0.3	3.0±0.5	0.407†
Loss of appetite	2.3±0.3	2.5±0.3	0.511†
Days with fever‡	0.24±0.1	1.0±0.3	0.017*
Total symptom score	79.3±7.4	102.5±12.2	0.056†

Values are mean ± SEM.
*P-value from independent Student's *t*-test.
†P-value from Mann–Whitney test.
‡Oral temperature >37.7°C.

participants who completed the study were available.

Symptoms

The sum of all symptoms recorded daily by questionnaires (total symptom score) was lower in the probiotic-treated group than in the control group. Differences were most marked in nasal, pharyngeal, and bronchial symptoms, as well as in the number of days with fever during a common cold episode. The other symptoms were also fewer but did not attain significance (Table 4).

Duration and incidence of episodes

The mean duration of common cold episodes was significantly shorter in the probiotic-treated group than in the control group (relative reduction of 21.5%, Table 3). The total number of common cold episodes was 158 in the probiotic-treated group and 153 in the control group (not significant).

Flow cytometric analysis

Volunteers with abnormal leucocyte counts before intervention were rejected from the analysis. After the consumption of probiotic bacteria for 14 days, a significantly higher enhancement, calculated as difference between day 14 and day 0, of cytotoxic T-cells plus T suppressor cells (CD8+) was observed compared to control. Also the enhancement of the other immune cells investigated was higher in the probiotic-treated group, but it was only marginally significant or not significant (Table 5). There was no change in T-cell activation and phagocytic activity during the observation period (data not shown).

Viral infections

Ninety-five samples of nasal secretion were obtained. Viruses were identified in 24 of these samples: rhinoviruses in 19, RS viruses in five and enterovirus (not specified) in one. In one sample, a dual viral infection was found.

Fecal lactobacilli and bifidobacteria

The mean absolute numbers of cells per g stool assessed in a separate trial were significantly increased from day 0 to day 14 days of probiotic supplementation for both the bifidobacteria ($3.14 \times 10^7 \pm 5.57 \times 10^6$ vs. $3.39 \times 10^8 \pm 5.53 \times 10^7$) and the lactobacilli ($1.04 \times 10^5 \pm 3.69 \times 10^4$ vs. $1.45 \times 10^5 \pm 2.48 \times 10^4$) (Fig. 1).

Discussion

In this randomized, placebo-controlled, double-blind intervention study the long term effect of probiotic bacteria on naturally acquired common respiratory tract infections was investigated. The winter/spring periods were chosen because of the enhanced risk of cold infections. Since viruses are known to vary between the common cold seasons the inclusion of two seasons, although done for technical reasons, served the aim of a representative study. The total number of common cold episodes registered in this study was 311 (0.7 episodes per participant within 4.3 months) which corresponds to the rule of thumb that adults catch two to four cold episodes per year.[1]

In this investigation, self-assessment based questionnaires were used.[13,14] This was justified by studies, which demonstrate a good concordance between the individual's own estimation of symptoms and an evaluation by a physician.[23]

Analysis of the questionnaires showed that the use of probiotic bacteria significantly shortened the mean duration of the episodes and reduced the

Table 5 Effect of probiotic bacteria (5×10^7 cfu/day) on cellular immune parameters. On day 0 and day 14 of the first intervention period immune cell counts were assessed by flow cytometry in blood samples from 122 participants (61 per study group). Volunteers with abnormal leucocyte counts before intervention were rejected from the analysis. Results are expressed as Δ (day 14–day 0) cell counts per μL blood (mean±SEM).

	(Δ Cell counts $\times 10^6$ per L blood)		P^*
	Probiotics+vitamins and minerals	Control (vitamins and minerals)	
Leukocytes	1749±556	1322±304	0.251
Lymphocytes	263±68	192±61	0.219
B lymphocytes	18±10	12±10	0.332
T lymphocytes	131±43	72±45	0.175
T_H	82±31	21±30	0.081
T_C+T_S	64±15	19±19	0.035
Natural killer cells	114±27	108±21	0.428
Granulocytes	1241±495	960±251	0.307
Monocytes	177±45	137±36	0.257

T_H, T helper cells (CD4+); T_C+T_S, cytotoxic T-cells+T suppressor cells (CD8+).
*P-values from independent Student's *t*-test.

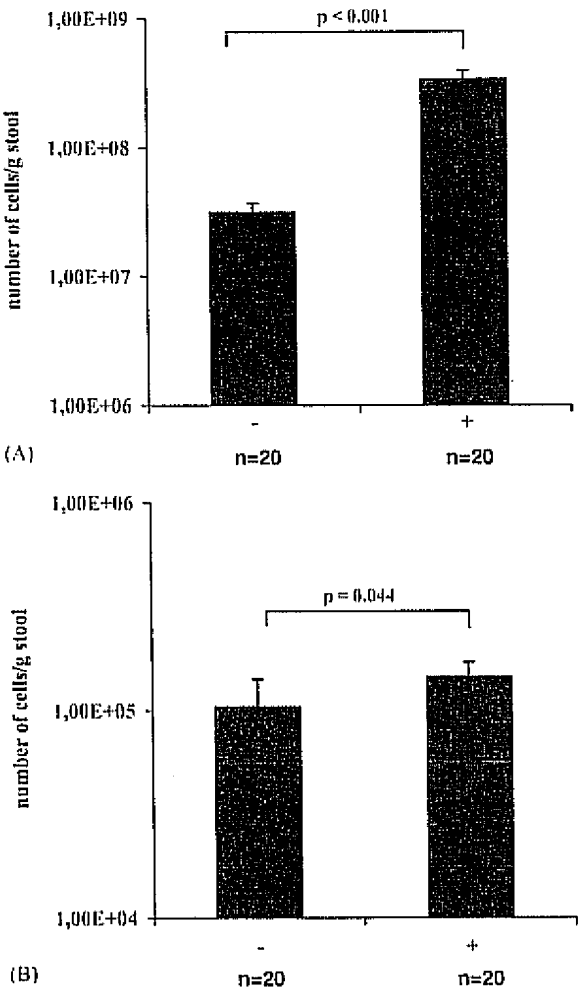

Figure 1 Effects on the number of bacterial cells of *Bifidobacterium* spp. (A) and *Lactobacillus* spp. (B) as assessed by quantitative real time PCR using group specific primers. Indicated are the mean absolute numbers of cells per g stool±SEM before (−) and after 14 days (+) of probiotic supplementation.

severity of symptoms. There was, however, no influence on the over all incidence of respiratory tract infections. The effects were similar to those of neuraminidase inhibitors in the case of acute influenza that, when administered within the first 48 h of the infection, reduced the severity of symptoms and shortened influenza by one day and, when inhaled prophylactically, by 2.5 days.[21,24] In the present study a reduction of about 2 days was found. In contrast to neuraminidase inhibitors, probiotics may be ingested as part of the daily diet and then do not induce extra costs to the health system.

The weak influenza-like symptoms and the mostly mild episodes of fever led to the conclusion that the infections were predominantly common cold episodes. This is also supported by the results of the PCR-analysis, which mainly detected rhinoviruses.

The reduction in the severity and duration of common cold episodes may be due to immune stimulatory effects. In response to certain probiotic strains, different cell lines such as human PBMCs, monocytes, dendritic cells, and human intestinal mucosa cells have been shown to release pro- and anti-inflammatory cytokines, as well as to express co-stimulatory molecules.[25–27] Such immunomodulation seems to be based on bacterial patterns, such as bacterial cell wall components (e.g. lipoteichoic acid) and CpG motifs of DNA (cytosine–phosphate–guanosine DNA, frequently found in bacterial and viral genome). They are recognized by toll-like receptors, which are expressed in intestinal and immune cells.[27–29] Moreover, several strains of *L. gasseri* have been shown to release a chemotactic factor acting on human monocytes.[30]

Cytotoxic T-cells plus T suppressor cells (CD8+) showed a significantly higher enhancement in the probiotic-treated group during the first 14 days of supplementation compared to the control group. By the probiotic bacteria used in the present study all immune cells investigated were enhanced. However, the enhancement was not significant or was only marginally significant. This may be due to the fact that both the probiotics and the control preparation contained supplemental vitamins and minerals, some of which have immunostimulatory properties; this could have blurred differences between the groups.[31]

The quantitative RT-PCR demonstrated a substantial increase of fecal lactobacilli and bifidobacteria after administration of the probiotic preparation. This may indicate survival during gastrointestinal transit, which is part of most of the definition proposed in literature.[32]

In conclusion, the present study presents evidence for the positive effects of consumption of *L. gasseri* PA 16/8, *B. longum* SP 07/3, *B. bifidum* MF 20/5 during at least 3 months in winter/spring on the severity of common cold episodes in otherwise healthy adults.

The growing number of studies in which favorable effects have been described, indicates some benefit of probiotics in respiratory tract infections. Until now the evidence, however, is based on one study for one strain or strain mixture, each. This has to be considered since probiotic effects are regarded as strain specific.

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Contributors: MV and JS contributed to the conception and design of the study and to all aspects of the study management. MV contributed further to the collection, statistical analysis and interpretation of the data and wrote the report with the critique and approval of the full research team. PW contributed to sample collection, care of volunteers, flow cytometric analyses, data collection and evaluation, and participated in writing the paper. PR contributed to the design of the study, the PCR analyses for viruses, and the statistical evaluation. CN and TH contributed to the PCR analyses for viruses. CL contributed to the clinical study. SO, JH, and SS contributed to the PCR analyses for fecal bacteria. KH contributed to the culture of probiotic bacteria and to questions in microbiology.

Conflict of interest statement: None declared. With the exception of funding limitations and the composition of the test preparations, the sponsors of the study (Merck Consumer Health Care) had no role in the study design, data collection, analysis and interpretation, or in the writing of the report.

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EXHIBIT B

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ORIGINAL ARTICLE

Probiotics prophylaxis in children with persistent primary vesicoureteral reflux

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Abstract Probiotics, beneficial living microorganisms, have been proven to be effective in preventing gastrointestinal infections, but their effect in preventing urinary tract infection (UTI) is inconclusive. A prospective randomized controlled study was done to compare the preventive effect of probiotics with conventional antibiotics in children with persistent primary vesicoureteral reflux (VUR). One hundred twenty children who had had persistent primary VUR after antibiotic prophylaxis for 1 year were randomly allocated into a probiotics (*Lactobacillus acidophilus* 10^8 CFU/g 1 g b.i.d., n=60) or an antibiotics (trimethoprim/sulfamethoxazole 2/10 mg/kg h.s., n=60) prophylaxis group during the second year of follow-up. The incidence of recurrent UTI was 18.3% (11/60) in the probiotics group, which was not different from 21.6%(13/60) in the antibiotic group (P= 0.926). The causative organisms of recurrent UTI were not significantly different between the two groups (P=0.938). Even after stratification by VUR grade, age, gender, phimosis, voiding dysfunction and renal scar, the incidence of recurrent UTI did not differ significantly between the two groups (P>0.05). The development of new renal scar was not significantly different between the two groups (P>0.05). In conclusion, probiotics prophylaxis was as effective as antibiotic prophylaxis in children with persistent primary VUR.

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Keywords Probiotics prophylaxis · *Lactobacillus acidophilus* · Recurrent UTI · Antibiotic prophylaxis

Introduction

Low-dose long-term antibiotic prophylaxis has been used during the past several decades to prevent urinary tract infections (UTI) in children with primary vesicoureteral reflux (VUR), in accordance with major VUR guidelines [1]. However, antibiotic prophylaxis did not significantly reduce the incidence of recurrent UTI in one randomized controlled study [2], and recent systemic reviews questioned the effect of antibiotic prophylaxis because of poor evidence [3, 4]. Recently, the emergence of resistant microorganisms has raised concerns about the long-term use of antibiotics [5], and probiotics have been deemed by the World Health Organization to be the next most important alternative regimen [6].

Probiotics, developed from the concept of normal flora, refers to beneficial live microorganisms when ingested in adequate amounts [7]. Lactic acid bacteria have been the common probiotic strains, because they exist naturally in human intestinal and urogenital tracts [8]. They are generally regarded as natural and safe and have been used to promote health for more than a century but have recently received more attention in preventing infection [9]. The preventive effects of probiotics have been proved in various gastrointestinal infections [10] and have been also promising in adult urogenital infections. Some well-characterized lactobacilli strains, oral or vaginal forms, improved vaginal ecology and prevented urogenital infection in women [11–16], even after antibiotics use [17, 18]. In children, human breast milk, known as natural probiotics, was proven to prevent UTI in infants [19], and commercially available

Table 1 Study children with persistent primary VUR after antibiotic prophylaxis in the first year

Characteristic	Probiotics	Antibiotics
Gender (male:female)	44:16	45:15
Age (month) (range)	19±12.1 (13–36)	21±11.4 (13–36)
VUR	6	7
Grade 1		
Grade 2	16	16
Grade 3	28	25
Grade 4	8	9
Grade 5	2	3
Total	60	60

(P>0.05)

probiotic products decreased the incidence of UTI in preterm infants [20]; additionally, there is one case report of successful prophylaxis in a girl with recurrent UTI [21]. However, there are not enough clinical data on the prevention of childhood UTI, and there is no clinical report that compares probiotics with antibiotics for preventing UTI in children with VUR.

We have prospectively compared the effect of probiotic prophylaxis with conventional antibiotic prophylaxis for preventing recurrent UTI in children with persistent primary VUR.

Patients and methods

One hundred twenty children with persistent primary VUR after antibiotic [trimethoprim-sulfamethoxazole (TMP/SMX)] prophylaxis for 1 year were prospectively randomized into a probiotic (n=60) or an antibiotic (n=60) group at Ewha Womans University Mokdong Hospital between 2002 and 2006. All children with primary VUR that had persisted after 1 year were included. Those with secondary VUR were excluded. The sample size in this study was less than the original calculated sample size ($2n$=125) for testing equivalency at α=0.05 and β=0.2 (power 80%). Thus, the calculated statistical power of 78% was slightly lower than the expected power, which would give some limitation of this study. The study protocol was approved by the ethics committee of the hospital, and informed consent was obtained from the parents of the study children. After stratified randomization, *Lactobacillus acidophilus* (antibio300 Hanwha Co., Korea, 1.0×10^8 CFU/g ATCC 4356) was given twice a day to the probiotic group and low-dose TMP/SMX (2/10 mg/kg) was given once a day before sleep to the antibiotic group. The differences in age (19±12.1 months vs 21±11.4 months), male-to-female ratio (44:16 vs 45:15) and the degrees of VUR were not significant between the probiotic and antibiotic groups (Table 1, P>0.05). Three children, two from the probiotic group and one from the antibiotic group, were not compliant, which was defined as having less than 80% of the prescribed drugs left over.

Recurrent UTI was diagnosed by significant bacteriuria in symptomatic children, such as fever, dysuria and pus in the diaper. Significant bacteriuria was defined by over 10^3 CFU/ml in supra-pubic aspirated urine culture or over 10^5 CFU/ml in catheterized urine culture (non-toilet trained children) and clean caught urine culture (toilet-trained children). The incidence of recurrent UTI was compared between the two study groups, and the difference in recurrent UTI rates between the two groups was also evaluated after being stratified by the well-known risk factors for UTI, such as VUR, age, gender, phimosis, voiding dysfunction and renal scar. Phimosis was diagnosed when the prepuce was not fully retractile. None of the male children had been circumcised. Voiding dysfunction was defined by the presence of frequency, urgency and/or incontinence in toilet-trained children. Renal scar was defined by ^{99m}Tc DMSA scan taken 3–6 months after their first DMSA(+) UTI. Development of new scar was also defined by ^{99m}Tc DMSA scan taken 3–6 months after recurrent DMSA(+) UTI.

Table 3 Causative organisms of recurrent UTI

Causative organism	Probiotics number (%)	Antibiotics number (%)
E. coli	7 (63.6)	9 (69.2)
Klebsiella pneumoniae	2 (18.2)	2 (15.4)
Enterobacter cloaca	1 (9.1)	1 (7.7)
Citobacter aerogens	–	1 (7.7)
Enterococcus faecalis	1 (9.1)	–
Total	11 (100)	13 (100)

(P=0.938)

Table 2 Incidence of recurrent UTI during prophylaxis in the second follow-up year in children with persistent primary VUR

Parameter	Probiotics n=60	Antibiotics n=60
Recurrent UTI, febrile	9 (15.0%)	11 (18.3%)
Recurrent UTI, afebrile	2 (3.3%)	2 (3.3%)
Total	11 (18.3%)	13 (21.6%)

(P=0.926)

Table 4 *E. coli* and its sensitivity to TMP/SMX in recurrent UTI

Sensitivity	Probiotics number (%)	Antibiotics number (%)
Sensitive	4 (57.1)	0 (0)
Resistant	3 (42.9)	9 (100)
Total	7 (100)	9 (100)

(P<0.019)

Table 5 Recurrence rate of UTI after stratification by VUR grade

Degree		Probiotics, number (%)	Antibiotics, number (%)	*P*
VUR	Grade 1	1/6 (16.7)	1/7 (14.3)	1.000
	Grade 2	2/16 (12.5)	1/16 (6.3)	1.000
	Grade 3	6/28 (21.4)	7/25 (28.0)	0.579
	Grade 4	1/8 (12.5)	3/9 (33.3)	0.576
	Grade 5	1/2 (50.0)	1/3 (33.3)	1.000
	Total	11/60 (18.3)	13/60 (21.6)	0.819

The results were analyzed in an 'intention-to-treat' model. For statistical analysis, the chi-square statistic and Fisher's exact test were conducted using SPSS version 11.0 for Windows. *P* values of less than 0.05 were considered significant.

Results

The incidence of recurrent UTI was 18.3% (11/60) in the probiotic group, which was not significantly different from 21.6% (13/60) in the antibiotic group (P=0.926). One episode of recurrent UTI developed in a non-compliant child in the antibiotic group. Most children with recurrence suffered only one episode, except one child in the antibiotic group, who had two episodes. Most recurrent UTIs were febrile in both groups (Table 2). The reason for the unusually high incidence of febrile UTI is obscure. However, one suggested reason is that the parents brought the children to the emergency room when they developed fever. The parents tended to take the children with mild symptoms to private clinics, where they were treated with antibiotics without urine culture. Thus, some afebrile UTIs might have been missed in this study. The causative organisms of recurrent UTI in the probiotic group were *Escherichia coli* (63.6%), *Klebsiella pneumoniae* (18.2%), *Enterobacter cloaca* (9.1%), *Enterococcus faecalis* (9.1%), which was not significantly different from *Escherichia coli* (69.2%), *Klebsiella pneumoniae* (15.4%), *Enterobacter cloaca* (7.7%), *Citobacter aerogens* (7.7%) in the antibiotic group (P=0.938) (Table 3). Sensitivity to TMP/SMX of *E. coli* in the probiotic group was 57.1% (4/7), which was significantly higher than 0% (0/9) in the antibiotic group (P<0.019) (Table 4).

Table 6 Recurrence rate of UTI after stratification by age

Age	Probiotics, number (%)	Antibiotics, number (%)	*P*
<2 years	9/45 (20.0)	9/44 (20.5)	0.957
>2 years	2/15 (13.3)	4/16 (25.0)	0.654

Table 7 Recurrence rate of UTI after stratification by gender

Gender	Probiotics, number (%)	Antibiotics, number (%)	*P*
Male	9/44 (20.5)	8/45 (17.7)	0.748
Female	2/16 (12.5)	5/15 (33.3)	0.220

As the study children were stratified according to their degree of VUR, the incidences of recurrent UTI were not statistically different between the two groups (P>0.05) (Table 5). Even after being stratified by age or gender, the incidences of recurrent UTI were not statistically different between the two groups (P>0.05) (Tables 6 and 7). In boys, most recurrent UTIs developed in those with phimosis. However, the incidences of recurrent UTIs, after being stratified by the presence of phimosis, were not significantly different between the two groups (P>0.05) (Table 8). In toilet-trained children, recurrent UTIs developed more in children with voiding dysfunction, but the incidences of recurrent UTIs after being stratified by the presence of voiding dysfunction were not significantly different between the two groups (P=1.000) (Table 9). Even after being stratified by the degree of renal scar, the incidences of recurrent UTIs were not statistically different between the two groups (P>0.05) (Table 10). Development of new renal scar after recurrent UTI was not statistically different between the two groups (P=0.595) (Table 11).

Discussion

In this randomized controlled study, probiotic *L. acidophilus* (2×10^8 CFU/day) was as effective as low-dose TMP/SMX in preventing recurrent UTI in the children with persistent primary VUR. The recurrence rate of UTI was 0.18/patient-year in the probiotic group and 0.22/patient-year in the antibiotic group, which was much less than the previously reported recurrence rate (0.6/patient-year) in UTI children with no prophylaxis [22].

The role of lactobacillus in reducing the risk of UTI originates from a study in 1915, which reported the successful treatment of cystitis by intravesical injections

Table 8 Recurrence rate of UTI after stratification by phimosis in boys

Presence	Probiotics, number (%)	Antibiotics, number (%)	*P*
Phimosis (+)	9/39 (23.1)	7/39 (17.9)	0.535
Phimosis (−)	0/5 (0)	1/6 (16.7)	1.000

Table 9 Recurrence rate of UTI after stratification by voiding dysfunction in toilet-trained children

Presence	Probiotics, number (%)	Antibiotics, number (%)	*P*
Voiding dysfunction (+)	1/4 (25.0)	2/6 (33.3)	1.000
Voiding dysfunction (−)	1/12 (8.3)	1/11 (9.1)	1.000

Table 11 Development of new renal scar after recurrent UTI

Presence	Probiotics, number (%)	Antibiotics, number (%)
New renal scar (−)	10 (90.9)	11 (84.6)
New renal scar (+)	1 (9.1)	2 (15.4)
Total	11 (100)	13 (100)

(P=0.596)

of *Lactobacillus* strains [23]. However, its role was ignored during the following antibiotics era. The first evidence of the importance of indigenous lactobacilli came from a study in 1973 that showed that vaginal lactobacilli in women patients with recurrent UTI were significantly depleted, in comparison with those in healthy control women [24]. A more recent study showed an inverse association of hydrogen peroxide (H_2O_2)-producing lactobacilli and vaginal *Escherichia coli* colonization in women with recurrent urinary tract infection [25]. Since then, there has been the view that exogenously applied lactobacilli, termed probiotics, would provide the same bacterial barrier to the uropathogens as the indigenous lactobacilli did.

Various lactobacillus isolates from dairy, poultry and human sources were tested for their abilities against uropathogens and were found to have anti-uro-pathogenic properties, which was widely variable, even within the same strain [26, 27]. In in vitro tests, lactobacilli could produce mucin, bacteriocin and a biosurfactant barrier, interfere with the uro-epithelial receptor sites with the adhesion of uro-pathogens, down-regulate inflammatory cytokines (IL-8, COX) or virulence factor expressions and up-regulate host immune responses (IgA, IL-10, IL-12) [28, 29]. More specifically, *L. acidophilus* was capable of inhibiting a test strain of *E. coli* in vitro and was considered to be a natural antibiotic [30]. *L. rhamnosus* GR-1 was highly adherent to uro-epithelial cells and inhibited growth and adhesion of uro-pathogens and *L. reuteri* (formerly *L. fermentum*) RC-14 produced a highly potent biosurfactant and hydrogen peroxide (H_2O_2) to inhibit the adhesion of uro-pathogens [31]. *L. acidophilus* NCFM produced a biosurfactant and H_2O_2 and inhibited uro-pathogens from adhering to uro-epithelial cells [32]. In an animal model, indigenous an *L. casei* strain, instilled into the bladder, vagina and urethra before challenge with uro-pathogens, prevented UTI in 84% [33] of cases, and *L. casei* Shirota strain eradicated *E coli*, ostensibly by modulation of host immune responses such as stimulation of natural killer cell activity [34].

Table 10 Recurrence rate of UTI after stratification by the degree of renal scar

Degree	Probiotics, number (%)	Antibiotics, number (%)	*P*
Renal scar (−)	5/33 (15.2)	7/36 (19.4)	0.638
Renal scar (+)	6/27 (22.2)	6/24 (25.0)	0.815
Segmental	2/19 (10.5)	3/17 (17.6)	0.650
Atrophic	4/8 (50.0)	3/7 (42.9)	1.000

In the human clinical trials, positive results have been obtained by using only well-characterized lactobacilli strains. The intravaginal instillation of *L. rhamnosus* GR-1 or *L. fermentum* RC-14 stimulated the indigenous vaginal lactobacilli and reduced the UTI recurrence rate from six to 1.6 episodes per year in women [12]. *L. rhamnosus* strain GR-1, originally isolated from the distal urethra of healthy woman, was more capable of colonizing the vagina and reducing the risk of UTI than was *L. rhamnosus* GG, originally isolated from feces [13]. The women consuming *L. acidophillus* yogurt at least three times per week had fewer episodes of UTI than those women who did not [14]. Daily oral intake of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 led to a significant reduction in uro-pathogens in the vaginas of healthy women [15]. A combination of *L. rhamnosus* GR-1 and *L. fermentum* RC-14, when applied as a suppository, colonized the human vagina more than *L. rhamnosus* GG did [16]. Vaginal suppositories of *L. rhamnosus* GR-1/*L. fermentum* RC14, administered with antibiotics in UTI, reduced the recurrence rate of UTI, which was similar to the recurrence rate in antibiotics prophylaxis [17]. A 10-day course of antibiotics plus 3 weeks of treatment with *L. rhamnosus* GR-1/*L. fermentum* RC14 suppositories was shown to maintain normal vaginal microbiota [18].

In children, the natural probiotic, human breast milk, decreased the incidence of urinary tract infection in infants [19]. Additionally, in premature infants given milk supplemented with *L. rhamnosus* GG, the episodes of UTI were reduced, although the difference was not statistically significant [20]. *L. acidophilus* DDS-1 capsules successfully prevented recurrent UTI in one 6-year old girl [21]. Those strain-to-strain differences of Lactobacilli remain to be elucidated [35].

The benefits of urogenital probiotics in childhood UTI include the avoidance of the side effects and emergence of resistant strains associated with long-term repeated use of antibiotics. Another advantage to patients is that it is

a natural approach that replenishes the depleted normal microflora to create a better environment to fight off uro-pathogens [36].

There are still many unresolved questions, such as what are the most effective strains, the ideal combination of strains, effective doses, safety of long-term use and the effect in children. However, promising evidence from the use of some probiotics in women with urogenital infections suggests possible benefits, even in childhood UTI. These were the rationale of studying probiotics as an alternative to antibiotics as prophylaxis to prevent UTI. For ideal urogenital probiotics, it is important to identify the best lactobacillus strains that are detrimental to uro-pathogens by further clinical trials.

In conclusion, this is the first randomized controlled study to show the role of probiotic *L. acidophilus* for preventing recurrent UTI in children with persistent primary VUR. However, this study has the limitation of lacking a treatment arm and a low calculated power (78%). We suggest further clinical trials to compare antibiotic prophylaxis with probiotics and no prophylaxis in children with or without primary VUR.

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EXHIBIT C

Prevention of Recurrence With Epirubicin and Lactobacillus Casei After Transurethral Resection of Bladder Cancer

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Purpose: A prospective, randomized, controlled trial was done to evaluate whether oral administration of a preparation of the probiotic agent Lactobacillus casei (Yakult Honsha, Tokyo, Japan) could enhance the prevention of recurrence by intravesical instillation of epirubicin after transurethral resection for superficial bladder cancer.

Materials and Methods: Between August 1999 and December 2002, 207 patients clinically diagnosed with superficial bladder cancer were included as study candidates and underwent transurethral resection, followed by intravesical instillation of 30 mg epirubicin/30 ml saline twice during 1 week. After histological confirmation of superficial bladder cancer they were again included as study participants with 102 randomized to receive treatment with 6 additional intravesical instillations of epirubicin during the 3-month period after transurethral resection (epirubicin group) and 100 randomized to intravesical chemotherapy on the same schedule as the epirubicin group plus oral administration of 3 gm Lactobacillus casei preparation per day for 1 year (epirubicin plus Lactobacillus casei group). Patients were evaluated for intravesical recurrence, disease progression, prognosis and adverse drug reactions.

Results: The 3-year recurrence-free survival rate was significantly higher in the epirubicin plus Lactobacillus casei group than in the epirubicin group (74.6% vs 59.9%, $p = 0.0234$), although neither progression-free nor overall survival differed between the groups. The incidence of adverse drug reactions did not significantly differ between the groups and there were no serious adverse drug reactions.

Conclusions: Intravesical instillation of epirubicin plus oral administration of Lactobacillus casei preparation is a novel, promising treatment for preventing recurrence after transurethral resection for superficial bladder cancer.

Key Words: bladder; bladder neoplasms; neoplasm recurrence, local; epirubicin; Lactobacillus casei

Although superficial bladder cancer can be treated with TUR, the high frequency of intravesical recurrence is a concern. It was reported that intravesical recurrence develops in 50% to 70% of patients within 5 years after TUR for superficial bladder cancer and the risk of progression to invasive cancer is 5% to 20%.[1] The recurrence risk peaks in the early postoperative phase at 100 to 120 days after TUR for multiple tumors and at 350 to 440 days after TUR for a solitary tumor, and then it decreases to and continues at a stable level for a long period.[2] Intravesical instillation therapy using anticancer agents or BCG has been developed to prevent intravesical recurrence of superficial bladder cancer after TUR. Intravesical instillation of mitomycin C, doxorubicin or EPI-ADM was reported to decrease the short-term (1 to 3-year) recurrence rate by about 20%.[3] On the other hand, intravesical instillation of BCG has stronger efficacy for preventing recurrence than anticancer agents, although the incidence and severity of adverse effects are higher with it than with chemotherapy.[4] Therefore, BCG is reserved for patients with high risk superficial bladder cancer, while anticancer agents are used in patients with intermediate risk cancer.

The LC preparation used in the study was a powdered preparation containing about 1×10^{10} cells of LC Shirota strain per gm. In Japan LC preparation has been safely used as a probiotic agent for more than 30 years. When it is orally administered, the LC preparation was reported to act as an immunomodulator through the intestinal tract and potentiate antitumor responses in mice.[5] Intravesical instillation of heat killed cells of the LC Shirota strain was also shown to exert antitumor effects in mice with bladder cancer and prevent bladder cancer.[6] In a randomized, comparative clinical trial Aso et al reported that the 50% recurrence-free interval after TUR for superficial bladder cancer was significantly prolonged by oral LC preparation to 1.8 times that in a control group.[7] They also performed a placebo controlled, double-blind clinical trial and noted that treatment with LC preparation was safe and effective for preventing intravesical recurrence after TUR for superficial bladder cancer.[8]

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For another article on a related topic see page 743.

These reports suggest that LC preparation can prevent intravesical recurrence after TUR for superficial bladder cancer through a mechanism different from that of intravesical chemotherapy.

Therefore, we planned a randomized, controlled trial in patients with superficial bladder cancer at intermediate risk for recurrence to evaluate whether postoperative oral administration of LC preparation could enhance the prevention of recurrence by intravesical instillation chemotherapy with EPI-ADM after TUR for superficial bladder cancer.

MATERIALS AND METHODS

This study was a multicenter, prospective, nonblinded, randomized, controlled trial. Patients were considered eligible if they had clinical stage Ta or T1, grade 1 or 2 primary or recurrent transitional cell carcinoma and the tumors appeared to have been eliminated completely by TUR. Patients meeting any of certain criteria were excluded from study, including a primary single Ta grade 1 tumor, grade 3 tumor, multiple recurrent tumors, history of urothelial carcinoma of the upper urinary tract, history of intravesical instillation of EPI-ADM or BCG, history of intravesical instillation of any agents during the 4-week period preceding the study, other active neoplasms or serious medical conditions. LC strain is contained in a fermented milk drink such as Yakult (Yakult Honsha, Tokyo, Japan) or a fermented milk product such as yogurt as an intestinal remedy. Therefore, patients regularly ingesting such beverage, food or drug containing LC strains were also excluded.

Before treatment patients provided a history and underwent physical examination, urinalysis, urine cytology examination, complete blood count, blood urea nitrogen and serum creatinine determination, liver function tests and electrocardiography. Chest x-rays and excretory urography were also performed. At study entry no patients had evidence of residual tumor on endoscopic examination and urine cytology.

Patients were added to the study via fax using a 2-step method at the Kyushu University Urological Oncology Group Data Center. After providing informed consent in writing patients were documented as study candidates before TUR for superficial bladder cancer. A 30 mg dose of EPI-ADM dissolved in 30 ml physiological saline was instilled into the bladder through a sterile catheter immediately after (within 2 hours) and 1 week after TUR. Patients were instructed not to void for 2 hours after instillation. When confirmed to be eligible based on the results of histopathological examination of resected tumor specimens, patients were again included as study participants and randomly assigned to receive treatment with 6 additional intravesical instillations of EPI-ADM during the 3-month period after TUR (EPI-ADM group) or intravesical chemotherapy on the same schedule as the EPI-ADM group plus 3 gm oral LC preparation per day (EPI-ADM plus LC group). Intravesical instillation of EPI-ADM was administered 3, 4, 6, 8, 10 and 12 weeks after TUR. The LC preparation dose was determined according to trials by Aso et al, in which the efficacy and safety of 3 gm oral LC per day were observed.[7,8] Figure 1 shows the group treatment schedules. Administration of the LC preparation was begun within 2 weeks after randomization and continued for 1 year.

In each groups urinalysis and cytological examination of urine samples were performed monthly for 3 months after TUR, every 3 months in the first 2 years and at 6-month intervals thereafter. Cystoscopy was performed every 3 months in the first 2 years and at 6-month intervals thereafter. Local and systemic toxicity was also monitored. Routine laboratory tests (hematology and biochemistry) were performed before, and 1, 3, 6, 9 and 12 months after TUR. All patients were followed at least 3 years and the treatment method after the first recurrence was at the discretion of each investigator. The severity of adverse reactions was assessed according to Common Terminology Criteria for Adverse Events, version 2.0.

The primary end point of this trial was the intravesical recurrence-free survival rate. Recurrence was defined as positive findings on cystoscopy or consecutive positive findings on urine cytology. Positive findings on cystoscopy were confirmed histologically by biopsy or TUR. Secondary end points were the progression-free survival rate, the overall survival rate, and the incidence and severity of adverse drug reactions. Progression was defined as muscle invasive bladder cancer or metastasis. Intravesical recurrence-free, progression-free and overall survival was defined as the interval from TUR to each event or the last followup without an event. Observation was concluded on January 31, 2006.

Sample size was calculated as described. Assuming that the 3-year recurrence-free survival rate would be 55% and 75% in the EPI-ADM and EPI-ADM plus LC groups, respectively, 92 patients per group were required to detect a 20%

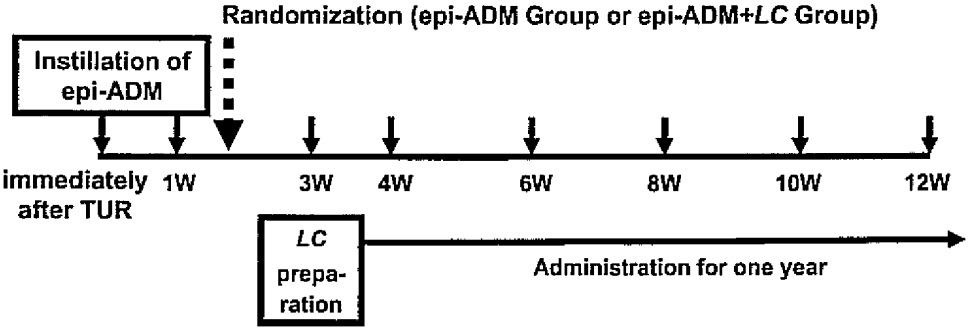

FIG. 1. Treatment schedule of EPI-ADM and EPI-ADM plus LC groups. In EPI-ADM group 30 mg EPI-ADM/30 ml saline were given immediately after (within 2 hours), and 1, 3, 4, 6, 8, 10 and 12 weeks (W) after TUR. In EPI-ADM plus LC group same intravesical instillation schedule was used as in EPI-ADM group plus oral administration of 3 gm LC preparation per day, which was begun within 2 weeks after randomization and continued for 1 year.

difference between the groups with a power of 80% and a 2-tailed significance level of 5%. Considering possible study dropouts due to inability to undergo the study, or violation of eligibility criteria or protocol treatment 200 study participants (100 per group) were set as the target sample size.

Statistical analysis was performed with an intent to treat design in all 202 eligible patients using the SAS® system package. The significance of differences in patient background factors, and the incidence and severity of toxicity between the 2 groups was examined by the chi-square test. Differences in time to first intravesical recurrence, progression or death were assessed using nonrecurrence, progression-free and overall survival curves, respectively, as calculated by the Kaplan-Meier method with the determination of statistical significance by the log rank test. The Cox proportional hazard model was used to adjust for possible bias in background factors. Differences were considered significant at $p < 0.05$. The risk of recurrence was defined as the fraction of patients with recurrence in each 6-month period. The HR in each period was calculated using maximum likelihood estimates derived from a piecewise exponential model.[9] The protocol was approved by the local institutional review board.

RESULTS

Between August 1999 and December 2002, 207 patients at 25 institutions were included as study candidates. They underwent TUR and subsequent intravesical instillation of EPI-ADM twice during 1 week. Five patients in whom histopathological review revealed invasive bladder cancer were eliminated from the study as ineligible. The remaining 202 patients were again documented as study participants and randomly assigned to the EPI-ADM group (102) or the EPI-ADM plus LC group (100). There were no significant differences between the groups in the sex ratio, patient age, smoking habit, number of tumors, T stage, grade or tumor size (table 1).

Participants were observed for recurrence for 0.6 to 79.9 (median 26.9) and 0.2 to 75.0 months (median 43.6) in the EPI-ADM and EPI-ADM plus LC groups, respectively. For progression they were observed for 0.6 to 79.9 (median 49.9) and 0.2 to 75.7 months (median 48.8), respectively. For any cause of death they were observed for 0.6 to 79.9 (median 49.9) and 0.2 to 75.7 months (median 49.1), respectively.

TABLE 1. *Patient characteristics*

	No. EPI-ADM	EPI-ADM + LC	p Value (chi-square test)
Overall	102	100	0.2510
Sex:			
M	86	78	
F	16	22	
Age:			0.8955
Younger than 70	55	53	
70 or Older	47	47	
Smoking habit:			0.6650
No	53	55	
Yes	49	45	
Tumor multiplicity:			0.9903
Primary/solitary	40	40	
Primary/multiple	52	50	
Recurrent/solitary	10	10	
T stage:			0.9955
Ta	53	52	
T1	49	48	
Tumor grade:			0.9425
1	21	21	
2	81	79	
Tumor size (cm):			0.8363
Less than 1	33	31	
1 or Greater	69	69	

More than 80% of patients in the EPI-ADM plus LC group ingested 80% or greater of the prescribed LC preparation throughout the 1-year treatment period, indicating high compliance with treatment with LC preparation. A total of 31 patients could not complete the protocol treatment irrespective of recurrence. One EPI-ADM group patient and 10 EPI-ADM plus LC group patients, including 4 during intravesical chemotherapy and 6 during treatment with LC preparation after intravesical chemotherapy, discontinued visiting the study institutions during the study period. Six and 14 patients in the EPI-ADM and EPI-ADM plus LC groups, respectively, discontinued study participation due to withdrawal of informed consent or exacerbation of other disease.

During the observation period bladder cancer recurred in 42 (41.2%) and 26 (26.0%) patients in the EPI-ADM and EPI-ADM plus LC groups, respectively. The 3-year recurrence-free survival rate was 59.9% (95% CI 49.9–69.8) in the EPI-ADM group and 74.6% (95% CI 65.5–83.6) in the EPI-ADM plus LC group. The EPI-ADM plus LC group showed a significantly higher recurrence-free survival rate than the EPI-ADM group (log rank test $p = 0.0234$, fig. 2).

To eliminate biases due to differences in background factors univariate analysis of 8 variables possibly affecting bladder cancer recurrence was performed using a Cox proportional-hazard model. Of the 8 variables group, multiplicity, tumor size and T stage each significantly affected the recurrence-free survival rate (table 2). On multivariate analysis the significance of the difference in the recurrence-free survival rate between the EPI-ADM and EPI-ADM plus LC groups was adjusted for the 3 other factors, ie multiplicity, tumor size and tumor stage. A significant difference between groups was also detected on multivariate analysis (table 3).

Figure 3 shows the risk of tumor recurrence during followup after TUR for superficial bladder cancer. In the EPI-ADM group the risk of tumor recurrence was increased in the first 2 years and it peaked around 6 months after TUR. However, in the EPI-ADM plus LC group the risk of tumor recurrence was clearly suppressed, especially in year 1 after TUR.

One and 3 patients in the EPI-ADM and EPI-ADM plus LC groups, respectively, showed disease progression. Death occurred during followup in 3 EPI-ADM group patients due to bladder cancer in 1 and other causes in 2, and in 4 EPI-ADM plus LC group patients due to bladder cancer in 1 and other causes in 3. There were no significant differences between the groups in progression-free or overall survival (data not shown).

Table 4 shows the incidence and severity of adverse reactions. The most common adverse reactions involved local toxicity due to intravesical chemotherapy, such as pain on micturition, urinary frequency and gross hematuria. In the EPI-ADM plus LC group 6% and 2% of patients had constipation and diarrhea possibly related to LC preparation treatment, respectively. There were no significant differences in the incidence of adverse reactions between the groups. Neither serious (grade 3 or 4) adverse reactions nor abnormal laboratory findings were observed in either group.

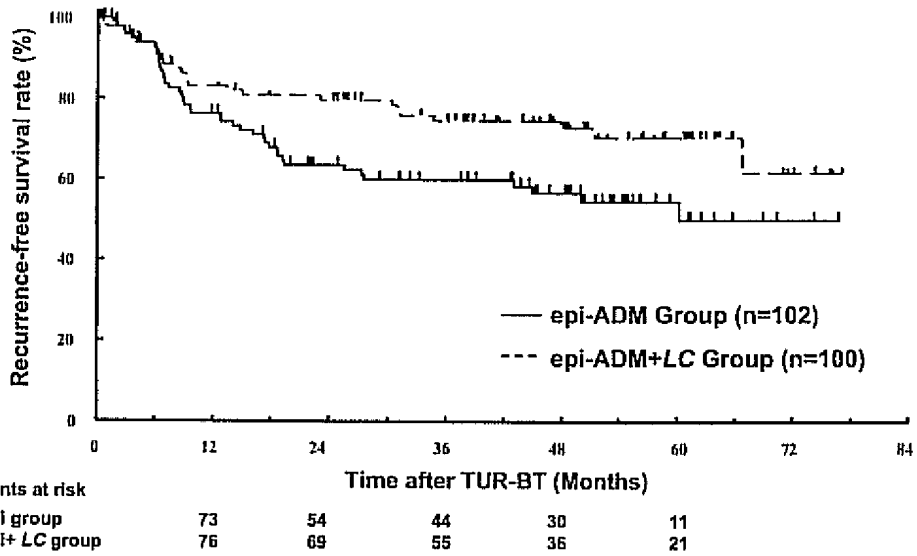

FIG. 2. Recurrence-free survival curves after TUR for superficial bladder cancer in 2 groups. EPI-ADM (*epi*) plus LC group showed significantly higher recurrence-free survival than EPI-ADM group (p = 0.0234).

No patients elected to discontinue protocol treatment due to adverse drug reactions.

DISCUSSION

Intravesical instillation chemotherapy with EPI-ADM is effective for preventing recurrence after TUR for superficial bladder cancer. Several reports suggest that sufficient preventive effects of EPI-ADM can be achieved even with relatively few instillations. In fact, Ali-El-Dein et al reported that single dose instillation of EPI-ADM immediately after TUR for superficial bladder cancer was as effective as multiple instillations of EPI-ADM for preventing tumor recurrence.[10] Okamura et al also reported that maintenance intravesical instillation of EPI-ADM did not improve the tumor recurrence rate.[11] However, in our previous study comparing 9 short-term and 19 long-term intravesical instillations of EPI-ADM long-term treatment was significantly more effective for preventing intravesical recurrence after TUR for superficial bladder cancer.[12] Thus, the optimal treatment schedule for intravesical EPI-ADM is still controversial. When considering cost-effectiveness and the risk of adverse reactions, a smaller number of intravesical instillations may be more beneficial. Therefore, 8 instillations of EPI-ADM, representing relatively short-term treatment, were performed in this trial.

Probiotics are viable bacteria that show beneficial health effects by improving the balance of intestinal bacterial flora.[13] The LC strain Shirota has been recognized as a typical probiotic strain.[14] A case-control study indicated that routine intake of Lactobacillus beverages may prevent bladder cancer.[15] Therefore, patients ingesting food, beverages or drugs containing Lactobacillus strains regularly were excluded from this study.

However, it is possible that EPI-ADM group patients began ingesting Lactobacillus products during this study, which may have resulted in underestimation of recurrence prevention by LC preparation. Regardless of this the EPI-ADM plus LC group showed significantly higher recurrence-free survival and the difference between the groups was still significant on multivariate analysis using factors found to be significant on univariate analysis.

Intravesical instillation of BCG is indicated to prevent recurrence after TUR for high risk superficial bladder cancer, although it sometimes causes severe toxicity. Since BCG was not used in this study, patients with a high risk tumor, such as a grade 3 tumor, or multiple recurrent tumors were excluded from study. However, it may be worthwhile to investigate the efficacy of LC preparation for preventing recurrence after TUR for such high risk superficial bladder cancers.

In the EPI-ADM plus LC group the risk of recurrence decreased in the early phase, especially in year 1 after TUR for superficial bladder cancer, in which the LC preparation was administered. The period of a clear suppression of the risk of tumor recurrence closely corresponded with that of the oral administration of LC preparation. Therefore, if LC

TABLE 2. *Univariate analysis of factors influencing recurrence*

Variables	HR	95% CI	p Value
Group (EPI-ADM + *LC*/EPI-ADM)	0.5714	0.3500–0.9328	0.0230
Age (70 or older/younger than 70)	1.3621	0.8463–2.1921	0.2034
Sex (F/M)	0.7135	0.3646–1.3964	0.3055
Smoking habit (yes/no)	1.1880	0.7383–1.9117	0.4776
Tumor multiplicity (multiple/single)	1.8342	1.1235–2.9945	0.0137
Tumor size (1 or greater/less than 1 cm)	1.8092	1.0192–3.2116	0.0331
Tumor grade (2/1)	0.9770	0.5420–1.7610	0.9387
Tumor stage (T1/Ta)	1.8550	1.1458–3.0031	0.0113

TABLE 3. *Multivariate adjusted recurrence HRs*

Variables + Models	HR	95% CI	p Value
Univariate	0.5714	0.3500–0.9328	0.0230
Adjusted for:			
Tumor multiplicity	0.5734	0.3510–0.9368	0.0241
Tumor size	0.5549	0.3396–0.9066	0.0169
Tumor stage	0.5721	0.3504–0.9341	0.0233
Tumor multiplicity, size + stage	0.5654	0.3450–0.9265	0.0216

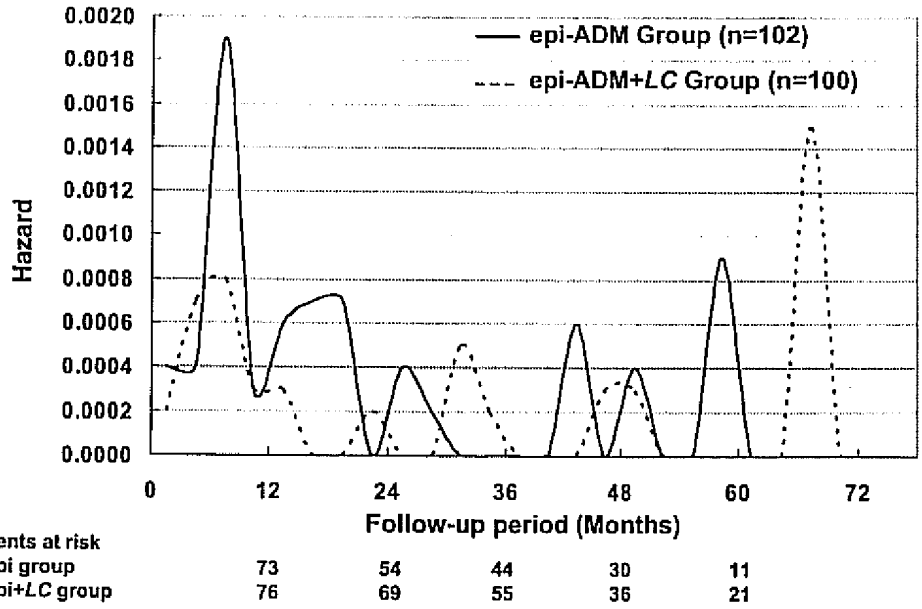

FIG. 3. Risk of tumor recurrence in EPI-ADM (*epi*) plus LC group was clearly decreased, especially in year 1 after TUR, compared to that in EPI-ADM group.

preparation oral administration were continued in the late phase after TUR, further improvement in recurrence-free survival may be achieved. Neither progression-free nor overall survival differed significantly between the groups, probably due to the small number of events. However, since intravesical recurrence may require hospital treatment, improvement in recurrence-free survival may be beneficial for decreasing costs for patients who must undergo TUR to treat superficial bladder cancer.

The LC strain contained in the LC preparation used is highly tolerant of gastric acid, bile acids and other components of digestive juices, and it can multiply in the intestinal tract to normalize intestinal flora. LC prevents the production and promotes the elimination of carcinogens and mutagens by intestinal bacteria, and it prevents the excretion of mutagens in urine in humans who have consumed fried ground beef.[16] Thus, LC is believed to prevent tumor development by improving intestinal flora. LC is also a biological response modifier that enhances immune system activity in humans.[17] It would be of great interest to clarify the mechanisms by which the combination of intravesical instillation of EPI-ADM and oral administration of LC preparation prevents intravesical recurrence after TUR for superficial bladder cancer.

The safety of LC preparation is established, although it may cause gastrointestinal symptoms in rare cases. While constipation and diarrhea developed in patients receiving LC preparation in the current study, the incidence was low, severity was mild and compliance with LC preparation treatment was favorable. There were no differences between the groups in the incidence or severity of adverse reactions, including those associated with micturition. Although the number of patients who could not complete the protocol treatment was higher in the EPI plus LC than in the EPI group (24 vs 7), this difference was not due to adverse reactions to LC preparation. Thus, the combination of intravesical instillation of EPI-ADM plus long-term oral LC preparation appeared highly tolerable.

Our findings reveal that adding oral treatment with LC preparation with a high degree of safety may enhance the efficacy of intravesical EPI-ADM for preventing intravesical recurrence after TUR for superficial bladder cancer. We are considering a prospective, randomized trial of this treatment

TABLE 4. *Adverse drug reaction incidence + severity*

	No. EPI-ADM (%)	No. EPI-ADM + LC (%)	p Value (chi-square test)
Pain on micturition:			
Grade 1	34 (33.3)	24 (24.0)	0.929
Grade 2	8 (7.8)	7 (7.0)	
Urinary frequency:			
Grade 1	22 (21.6)	19 (19.0)	0.905
Grade 2	9 (8.8)	6 (6.0)	
Gross hematuria:			
Grade 1	15 (14.7)	14 (14.0)	0.836
Grade 2	4 (4.0)	2 (2.0)	
Constipation:			
Grade 1	2 (2.0)	4 (4.0)	0.895
Grade 2	2 (2.0)	2 (2.0)	
Diarrhea:			
Grade 1	0	1 (1.0)	1.000
Grade 2	0	1 (1.0)	

vs BCG to prevent recurrence after TUR for superficial bladder cancer.

CONCLUSIONS

Oral LC preparation enhanced the efficacy of intravesical EPI-ADM for preventing recurrence after TUR for superficial bladder cancer. To our knowledge this is the first report of the additive effect of an oral nonchemotherapy for preventing tumor recurrence by intravesical chemotherapy.

Abbreviations and Acronyms

BCG	=	bacillus Calmette-Guerin
EPI-ADM	=	epirubicin
LC	=	Lactobacillus casei
TUR	=	transurethral resection

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EXHIBIT D

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Probiotic modulation of symbiotic gut microbial–host metabolic interactions in a humanized microbiome mouse model

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The transgenomic metabolic effects of exposure to either *Lactobacillus paracasei* or *Lactobacillus rhamnosus* probiotics have been measured and mapped in humanized extended genome mice (germ-free mice colonized with human baby flora). Statistical analysis of the compartmental fluctuations in diverse metabolic compartments, including biofluids, tissue and cecal short-chain fatty acids (SCFAs) in relation to microbial population modulation generated a novel top-down systems biology view of the host response to probiotic intervention. Probiotic exposure exerted microbiome modification and resulted in altered hepatic lipid metabolism coupled with lowered plasma lipoprotein levels and apparent stimulated glycolysis. Probiotic treatments also altered a diverse range of pathways outcomes, including amino-acid metabolism, methylamines and SCFAs. The novel application of hierarchical-principal component analysis allowed visualization of multicompartmental transgenomic metabolic interactions that could also be resolved at the compartment and pathway level. These integrated system investigations demonstrate the potential of metabolic profiling as a top-down systems biology driver for investigating the mechanistic basis of probiotic action and the therapeutic surveillance of the gut microbial activity related to dietary supplementation of probiotics.
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Keywords: metabonomics; microbiome; NMR spectroscopy; probiotics; UPLC-MS

Introduction

The gut microbiome–mammalian 'Superorganism' (Lederberg, 2000) represents a level of biological evolutionary development in which there is extensive 'transgenomic' modulation of metabolism and physiology that is a characteristic of true symbiosis. By definition, superorganisms contain multiple cell types, and the coevolved interacting genomes can only be effectively studied as an *in vivo* unit *in situ* using top-down systems biology approaches (Nicholson, 2006; Martin *et al*, 2007a). Interest in the impact of gut microbial activity on human health is expanding rapidly and many mammalian–microbial associations, both positive and negative, have been reported (Dunne, 2001; Verdu *et al*, 2004; Nicholson *et al*, 2005; Gill *et al*, 2006; Ley *et al*, 2006). Mammalian–microbial symbiosis can play a strong role in the metabolism of endogenous and exogenous compounds and can also be influential in the etiology and development of several diseases, for example insulin resistance (Dumas *et al*, 2006), Crohn's disease (Gupta *et al*, 2000; Marchesi *et al*, 2007), irritable bowel syndrome (Sartor, 2004; Martin *et al*, 2006), food allergies (Bjorksten *et al*, 2001), gastritis and peptic ulcers (Warren, 2000; Marshall, 2003), obesity (Ley *et al*, 2006; Turnbaugh *et al*, 2006), cardiovascular disease (Pereira and Gibson, 2002) and gastrointestinal cancers (Dunne, 2001). Activities of the diverse gut microbiota can be highly specific and it has been reported that the establishment of *Bifidobacteria* is important for the development of the immune system and

for maintaining gut function (Blum and Schiffrin, 2003; Salminen *et al*, 2005; Ouwehand, 2007). In particular, elevated counts in *Bifidobacterium* with reduced *Escherichia coli*, streptococci, *Bacteroides* and clostridia counts in breast-fed babies compared to formula-fed neonates may result in the lower incidence of infections, morbidity and mortality in breast-fed infants (Dai *et al*, 2000; Kunz *et al*, 2000). As the microbiome interacts strongly with the host to determine the metabolic phenotype (Holmes and Nicholson, 2005; Gavaghan McKee *et al*, 2006) and metabolic phenotype influences outcomes of drug interventions (Nicholson *et al*, 2004; Clayton *et al*, 2006), there is clearly an important role of understanding these interactions as part of personalized healthcare solutions (Nicholson, 2006).

One of the current approaches used to modulate the balance of intestinal microflora is based on oral administration of probiotics. A probiotic is generally defined as a 'live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 2004). The gastrointestinal system is populated by potentially pathogenic bacteria that are capable of degrading proteins (putrefaction), releasing ammonia, amines and indoles, which in high concentrations can be toxic to humans (Cummings and Bingham, 1987). Probiotic supplementation aims at replacing or reducing the number of potentially harmful *E. coli* and *Clostridia* in the intestine by enriching the populations of gut microbiota that ferment carbohydrates and that have little proteolytic activity. Probiotics, most commonly *Lactobacillus* and *Bifidobacteria*, can be used to modulate the balance of the intestinal microflora in a beneficial way (Collins and Gibson, 1999). Although Lactobacilli do not predominate among the intestinal microflora, their resistance to acid conditions and bile salts toxicity results in their ubiquitous presence throughout the gut (Corcoran *et al*, 2005), hence they can exert metabolic effects at many levels. Fermented dairy products containing *Lactobacillus* have traditionally been used to modulate the microbial ecology (Dunne, 2001). In particular, *L. paracasei* was shown to modulate the intestinal physiology, to prevent infection of pathogenic bacteria (Sarker *et al*, 2005), to stimulate the immune system (Ibnou-Zekri *et al*, 2003), and to normalize gastrointestinal disorders (Martin *et al*, 2006). *L. rhamnosus* is also a significant probiotic strain with proven health benefits and therapeutic applications in the treatment of diarrhea (Szynanski *et al*, 2006), irritable bowel syndrome (Kajander *et al*, 2005), atopic eczema (Corcoran *et al*, 2005) and the prevention of urinary tract infections (Reid and Bruce, 2006). However, the functional effects of probiotic interventions cannot be fully assessed without probing the biochemistry of the host at multiple compartmental levels, and we propose that top-down systems biology provides an ideal approach to further understanding in this field. The microbiota observed in human baby flora (HBF) mice have a number of similarities with that found in formula-fed neonates (Mackie *et al*, 1999), which makes it to be a well-adapted and simplified model to assess probiotics impact on gut microbial functional ecosystems (in particular on metabolism of *Bifidobacteria* and potential *pathogens*) and subsequent effects on host metabolism.

Metabolic profiling using high-density data generating spectroscopic techniques, in combination with multivariate mathematical modelling is a tool which is well suited to generate metabolic profiles that encapsulate the top-down system response of an organism to a stressor or intervention (Nicholson and Wilson, 2003). Multivariate metabolic profiling offers a practical approach to measuring the metabolic endpoints that link directly to whole system activity and which are determined by both host genetic and environmental factors (Nicholson *et al*, 2005). Recently, metabolic profiling strategies have been successfully applied to characterizing the metabolic consequences of nutritional intervention (Rezzi *et al*, 2007; Wang *et al*, 2007) the effects of the gut microflora on mammalian metabolism (Martin *et al*, 2006, 2007a, b) and mechanisms of insulin-resistance (Dumas *et al*, 2006). In the current study, ^{1}H nuclear magnetic resonance (NMR) spectroscopy and targeted ultra performance liquid chromatography-mass spectrometry (UPLC-MS) analysis have been applied to characterize the global metabolic responses of humanized microbiome mice subsequently exposed to placebo, *Lactobacillus paracasei* or *Lactobacillus rhamnosus* supplementation. Correlation of the response across multiple biofluids and tissue, using plasma, urine, fecal extracts, liver tissues and ileal flushes as the biological matrices for the detection of dietary intervention, generates a top-down systems biology view of the response to probiotics intervention.

Results

Gut bacterial composition

Microbiological analyses were performed on fecal samples to assess the growth of the HBF in germ-free mice and to ascertain the effects of probiotics on the development of gut bacteria. The measured terminal composition of the fecal microbiota is detailed in Table I, where the statistically significant differences between the various groups were calculated using a two-tailed Mann–Whitney test. The bacterial populations of *Bifidobacteria longum* and *Staphylococcus aureus* were reduced after introduction of both probiotics. Additionally, unique effects of *L. rhamnosus* supplementation caused decreased populations of *Bifidobacterium breve*, *Staphylococcus epidermidis* and *Clostridium perfringens* but an increase of *E. coli*.

Gut levels of short-chain fatty acids

Short-chain fatty acids (SCFAs), namely acetate, propionate, isobutyrate, *n*-butyrate and isovalerate, were identified and quantified from the cecal content using GC-FID. The results, presented in Table II, are given in μmol per gram of dry fecal material and as mean ± s.d. for each group of mice. The production of some of the SCFAs, that is, acetate and butyrate, by the HBF mice supplemented with both of the probiotics was reduced. In addition, increases of the concentrations in isobutyrate and isovalerate were observed in the mice fed with *L. paracasei*.

Analysis of ^{1}H NMR spectroscopic data on plasma, urine, liver and fecal extracts

A series of pairwise O-PLS-DA models of ^{1}H NMR spectra were performed to extract information on the metabolic effects of

Table I Microbial species counts in mouse feces at the end of the experiment

Groups/$\log_{10}$ CFU	HBF ($n = 10$)	HBF + *L. paracasei* ($n = 9$)	HBF + *L. rhamnosus* ($n = 9$)
L. paracasei	—	8.5 ± 0.2	—
L. rhamnosus	—	—	7.8 ± 0.2
E. coli	9.2 ± 0.3	9.4 ± 0.3	9.8 ± 0.5**
B. breve	9.1 ± 0.2	7.78 ± 2.13	8.7 ± 0.3*
B. longum	8.2 ± 0.6	5.6 ± 1.9***	6.3 ± 0.5***
S. aureus	7.4 ± 0.3	6.3 ± 0.3***	6.6 ± 0.5***
S. epidermidis	4.8 ± 0.4	4.9 ± 1.2	4.0 ± 0.5**
C. perfringens	7.2 ± 0.3	7.0 ± 0.5	5.7 ± 1.0***
Bacteroides	10.3 ± 0.2	10.4 ± 0.2	10.1 ± 0.4

$\log_{10}$ CFU (colony-forming unit) given per gram of wet weight of feces. Data are presented as mean ± s.d. Absence of specific bacterial strains in the gut microflora is indicated by "—". The values for the HBF mice supplemented with probiotics were compared to HBF control mice, *, ** and *** indicate a significant difference at 95, 99 and 99.9% confidence levels, respectively.

Table II Short-chain fatty acid content in the cecum from the different groups

Amounts of SCFAs given in μmol per gram of dry feces for each group	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate
HBF ($n = 10$)	77.6 ± 17.6	22.3 ± 4.3	0.9 ± 0.2	3 ± 0.6	2.1 ± 0.6
HBF + *L. paracasei* ($n = 9$)	52.3 ± 23.6***	22.2 ± 10.8	1.2 ± 0.5***	1.5 ± 0.8***	2.7 ± 1.2**
HBF + *L. rhamnosus* ($n = 9$)	40.6 ± 8***	20.3 ± 2.8	0.8 ± 0.2	2.1 ± 0.4***	2.1 ± 0.5

Data are presented in μmol per gram of dry feces and are presented as means ± s.d. The amounts of SCFAs for the HBF mice supplemented with probiotics were compared to HBF control mice, ** and *** indicate a significant difference at 99 and 99.9% confidence levels, respectively.

probiotic modulation. A statistically significant metabolic phenotype separation between untreated mice and probiotic supplemented animals was observed as reflected by the high value of Q^2_Y for each model (Cloarec *et al*, 2005b; Table III). The corresponding coefficients describing the most important metabolites in plasma, liver, urine and fecal extracts that contributed to group separation are also listed in Supplementary Table 1. The area normalized intensities (10^1 a.u.) of representative metabolite signals are given as means ± s.d. in Table III. The O-PLS-DA coefficients plots are presented in Figure 1 using a back-scaling transformation and projection to aid biomarker visualization (Cloarec *et al*, 2005b). The direction of the signals in the plots relative to zero indicates positive or negative covariance with the probiotic-treated class. Each variable is plotted with a color code that indicates its discriminating power as calculated from the correlation matrix thus highlighting biomarker-rich spectral regions.

Liver metabolic profiles

Livers of mice fed with *L. paracasei* showed relative decreases in dimethylamine (DMA), trimethylamine (TMA), leucine, isoleucine, glutamine, and glycogen and increased levels of succinate and lactate (Figure 1A). Mice supplemented with *L. rhamnosus* showed relative decreases in leucine and isoleucine and relative increases in succinate, TMA and trimethylamine-*N*-oxide (TMAO) in the liver compared to controls (Figure 1D).

Plasma metabolic profiles

Plasma samples showed relative decreases in the levels of lipoproteins and increases in the concentrations of glycerophosphorylcholine (GPC) and triglycerides in mice fed with both probiotics compared to controls (Figure 1B and E). Elevated choline levels were observed in plasma of mice fed with *L. rhamnosus* and reduced plasma citrate levels were observed in mice fed with *L. paracasei* compared to controls.

Fecal extract metabolic profiles

Marked changes were observed in the metabolic profiles of fecal extracts from all supplemented mice, for example relative decreased concentrations of choline, acetate, ethanol, a range of putative *N*-acetylated metabolites (NAMs), unconjugated bile acids (BAs) and tauro-conjugated bile acids (Figure 1C and F). Furthermore, relative higher levels of glucose, lysine and polysaccharides were detected in the feces from mice fed with probiotics. A relative increased level of n-caproate (chemical shifts δ at 0.89(t), 1.27(m), 1.63(q), 2.34(t)) appeared to be associated with mice supplemented with *L. paracasei*.

Urine metabolic profiles

Urine samples of mice supplemented with both probiotics showed relative increased concentrations of indoleacetylglycine (IAG), phenylacetylglycine (PAG), tryptamine and a relative decrease in the levels of α-keto-isocaproate and citrate (Figure 1G and H). Relative increased concentrations of a mixture of putative glycolipids (UGLp, chemical shifts of multiplets at δ 0.89, 1.27, 1.56, 1.68, 2.15, 2.25, 3.10, 3.55, 3.60), *N*-acetyl-glycoproteins (NAGs) and a reduction in 3-hydroxy-isovalerate were also observed in mice supplemented with *L. paracasei* compared to controls. Urine of mice fed with *L. rhamnosus* showed a reduction in levels of creatine and citrulline.

Table III Summary of influential metabolites for discriminating NMR spectra of liver, plasma, fecal extracts and urine

Metabolites	Chemical shift and multiplicity	HBF controls	HBF + *L. paracasei*	HBF + *L. rhamnosus*
Liver			$Q^2_Y = 21\%$, $R^2_X = 44\%$	$Q^2_Y = 41\%$, $R^2_X = 37\%$
Leu	0.92(t)	2.4 ± 0.6	1.7 ± 0.3***	1.9 ± 0.5*
Ileu	0.94 (t)	0.8 ± 0.1	0.6 ± 0.05***	0.7 ± 0.2
Lactate	1.32(d)	38.4 ± 5.8	46.2 ± 8.2*	39.2 ± 9.7
Succinate	2.41(s)	0.2 ± 0.1	1.0 ± 0.6**	0.7 ± 0.3*
MA	2.61(s)	0.1 ± 0.06	0.04 ± 0.002**	0.08 ± 0.05
TMA	2.91 (s)	0.2 ± 0.04	0.07 ± 0.03***	0.2 ± 0.09
TMAO	3.27(s)	10.3 ± 2.2	13.1 ± 3.7	18.5 ± 8.0**
Gln	2.44(m)	0.4 ± 0.1	0.3 ± 0.1*	0.3 ± 0.1
Glycogen	5.38–5.45	3.4 ± 1.9	1.5 ± 0.6*	3.2 ± 1.9
Plasma			$Q^2_Y = 44\%$, $R^2_X = 50\%$	$Q^2_Y = 51\%$, $R^2_X = 32\%$
Lipoproteins	0.84 (m)	13.7 ± 1.8	10.1 ± 0.9***	9.8 ± 4.2**
Citrate	2.65(d)	1.4 ± 0.3	0.9 ± 0.4**	1.1 ± 0.2**
Choline	3.2(s)	11.6 ± 2.6	16.2 ± 5.7*	20.5 ± 3.8***
GPC	3.22(s)	44.1 ± 4.6	57.3 ± 12.5**	68.1 ± 11.2***
Glyceryls	3.91(m)	2.0 ± 0.3	2.5 ± 0.4**	2.7 ± 0.4**
Feces			$Q^2_Y = 90\%$, $R^2_X = 48\%$	$Q^2_Y = 89\%$, $R^2_X = 49\%$
Caprylate	1.27(m)	2.5 ± 0.1	3.5 ± 0.2***	2.4 ± 0.1
Lys	3.00(m)	3.2 ± 0.8	5.0 ± 0.3***	4.9 ± 1.2**
Osides	5.42(m)	0.9 ± 0.09	1.2 ± 0.1***	1.4 ± 0.1***
Bile acids	0.72(s)	3.1 ± 0.9	1.8 ± 0.7**	2.0 ± 0.6*
Ethanol	1.18(t)	2.5 ± 0.1	2.0 ± 0.08***	1.9 ± 0.09***
Choline	3.20(s)	48.0 ± 19.5	11.3 ± 4.1***	20.3 ± 10.9***
NAM	2.06(m)	7.1 ± 1.0	5.4 ± 0.3***	5.5 ± 0.3***
Acetate	1.91(s)	58.7 ± 34.2	27.0 ± 9.2**	32.9 ± 12.9*
UI	3.71(s)	9.2 ± 0.5	7.3 ± 0.3***	8.2 ± 0.5***
Urine			$Q^2_Y = 91\%$, $R^2_X = 47\%$	$Q^2_Y = 59\%$, $R^2_X = 46\%$
IAG	7.55(d)	0.1 ± 0.03	0.6 ± 0.2***	0.4 ± 0.2**
PAG	7.37(m)	0.8 ± 0.1	1.5 ± 0.3***	1.2 ± 0.4*
Tryptamine	7.70(d)	0.1 ± 0.04	0.4 ± 0.1***	0.2 ± 0.1**
UGLp	1.27(m)	1.7 ± 0.1	2.7 ± 0.4***	1.7 ± 0.2
Glycero-metabolites	4.04 (m)	1.7 ± 0.1	2.2 ± 0.2***	1.9 ± 0.2*
NAG	2.04(s)	3.5 ± 0.2	4.3 ± 0.2***	3.8 ± 0.5
Butyrate	0.90(t)	6.9 ± 0.8	5.2 ± 0.7**	5.2 ± 0.9**
α-keto-isocaproate	0.94(d)	13.8 ± 4.6	6.1 ± 2.3**	7.9 ± 2.1**
Propionate	1.05(t)	0.9 ± 0.2	0.8 ± 0.04*	0.8 ± 0.1
3-hydroxy-isovalerate	1.24(s)	3.0 ± 0.4	2.1 ± 0.4**	2.6 ± 0.2
Citrate	2.55(d)	10.8 ± 7.6	1.6 ± 0.6**	2.2 ± 0.9*
Creatine	3.92(s)	5.7 ± 2.1	4.5 ± 1.5	3.5 ± 0.3**
Citrulline	1.88(m)	3.8 ± 0.5	3.3 ± 0.4	3.0 ± 0.4**

O-PLS models were generated for comparing probiotics treated to HBF control mice using one predictive and two orthogonal components, R_X^2 value shows how much of the variation is explained, Q_Y^2 value represents the predictability of the models, and relates to its statistical validity. Data are presented as area normalized intensities (10^1 a.u.) of representative metabolite signals as means ± s.d. The values for the HBF mice supplemented with probiotics were compared to HBF control mice, *, **, ** and *** indicate a significant difference at 90, 95, 99 and 99.9% confidence levels, respectively.
s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets. For metabolite abbreviations, refer to the key in Figure 1, MA: methylamine.

UPLC-MS analysis of bile acids in ileal flushes

The proportion of bile acids in ileal flushes from the different groups are given in Table IV and are shown as mean ± s.d. of the percentage of the total bile acid content. O-PLS-DA of the data set revealed that the relative concentrations of bile acids obtained from unsupplemented HBF mice are separated from those treated with probiotics, the correlation observed with *L. paracasei* being more significant than with *L. rhamnosus* as noted by the values of the cross-validated model parameter Q^2_Y (Figure 2). For example, HBF mice supplemented with *L. paracasei* showed strong correlations with higher amounts of GCA, CDCA and UDCA and lower levels of α-MCA in the ileal flushes when compared to controls. HBF mice fed with *L. rhamnosus* also showed higher levels of GCA associated with lower levels of TUDCA and TCDCA in the ileal flushes when compared to untreated HBF mice.

Integration of multicompartment metabolic data using hierarchical-principal component analysis

A principal component analysis (PCA) model was initially constructed separately for the metabolic data from each individual biological matrix (plasma, urine, liver, fecal extracts and bile acid composition in ileal flush; Figures 3 and 4). Three principal components were calculated for each cross-validated PCA model, except for the plasma where four principal components were retained to maximize the explained variance R^2X and the cross-validation parameter Q^2 following the standard sevenfold cross-validation method (Cloarec *et al*, 2005b). These PCA models descriptors (R^2X/Q^2) were 0.70/0.36 (plasma), 0.41/0.11 (urine), 0.80/0.71 (liver), 0.94/0.67 (bile acid) and 0.61/0.42 (feces). The score vectors t_b from each model were then assigned as new X-variables (Figure 3). Thus, the top level X-matrix contained 16 descriptors, denoted

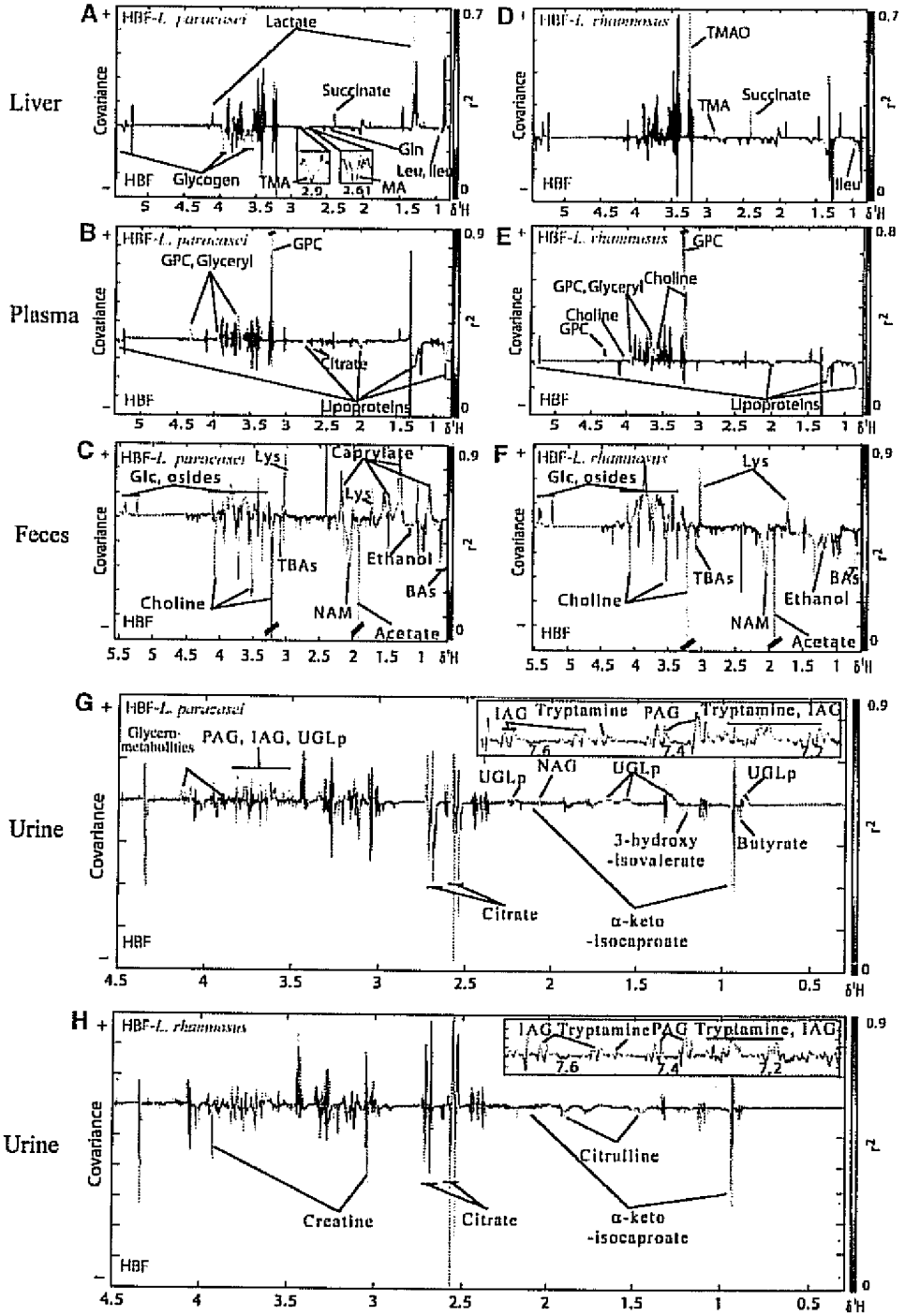

Figure 1 O-PLS-DA coefficient plots derived from ^{1}H MAS NMR CPMG spectra of liver (**A, D**), ^{1}H NMR CPMG spectra of plasma (**B, E**), ^{1}H NMR standard spectra of fecal extracts (**C, F**) and urine (**G, H**), indicating discrimination between HBF mice fed with probiotics (positive) and HBF control mice (negative). The color code corresponds to the correlation coefficients of the variables with the classes. BAs, Bile acids; DMA, dimethylamine; Glc, glucose; Gln, glutamine; GPC, glycerophosphorylcholine; IAG, indoleacetylglycine; Ileu, isoleucine; Leu, leucine; Lys, lysine; NAG, *N*-acetylated glycoproteins; NAM, *N*-acetylated metabolites; Osides, glycosides; PAG, phenylacetylglycine; TBAs, taurine conjugated to bile acids; TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide; UGLp, unidentified glycolipids.

P_i (plasma PCs 1–4), L_i (liver PCs 1–3), U_i (urine PCs 1–3), B_i (bile acid PCs 1–3) and F_i (fecal PCs 1–3), which comprise only the systematic variation from each of the blocks/compartments. The two first principal components (p1 and p2) calculated for the hierarchical-principal component analysis (H-PCA; Westerhuis *et al*, 1998) model (R^2X=0.60) accounted for 37 and 23% of the total variance in the combined multi compartment data respectively. The cross validation for the H-PCA model failed due to the high degree of orthogonality within the X-matrix, that is, within each of the blocks all variables

are orthogonal to each other, while each of the biological matrices could be cross-validated at the individual level.

The H-PCA scores plot illustrated a degree of clustering with respect to the groups of HBF mice (Figure 4A). The corresponding H-PCA loadings plot indicated the contribution of the 16 descriptors to the differences observed between the samples in the H-PCA scores plot. Probiotic-supplemented HBF animals are separated from the controls along the first principal component, and this arises from the main variations modelled at the base level PCA of the individual plasma (P1, P2), liver (L2, L3), ileal flush (B2) and urine (U1, U2) data sets (Figure 4B). HBF mice fed with *L. paracasei* were separated from those fed with *L. rhamnosus* along the second principal component, which was mainly due to the variations modelled at the base level PCA of plasma (P2, P3), liver (L1) and urine (U1, U3). Interestingly, the metabolic variations in the fecal samples have no weight in discriminating the bacterial supplementation from the corresponding controls in the global model.

To uncover variables contributing to the H-PCA super scores, the loadings at the base level PCA model were interrogated (Figure 4C–F). HBF mice supplemented with probiotics showed higher concentrations of glucose, choline, GPC, glutamine, glutamate and lysine in the plasma profiles associated with elevated concentrations of glucose in the liver and higher levels of TUDCA and TCDCA in the ileal flushes. Controls showed higher levels of lipoproteins in the plasma, elevated concentrations of lipids, glycogen, glutamine, glutamate, alanine, TMAO and lactate in the liver, associated with higher levels of TCA and TβMCA in the ileal content. Unsupplemented HBF mice also showed elevated urinary excretions of creatine, citrate, citrulline, lysine, UGLp, NAG and α-keto-isocaproate compared to animals fed with probiotics. Moreover, H-PCA also revealed that HBF mice treated with *L. rhamnosus* had higher levels of hepatic lipids and plasma lipoproteins but lower concentrations of lactate and amino acids in plasma and lower urinary excretion of PAG, IAG, tryptamine and taurine than HBF mice treated with *L. paracasei*.

Table IV Bile acids composition in gut flushes for the different microbiota

Microbiota/ Bile acids	HBF	HBF + *L. paracasei*	HBF + *L. rhamnosus*
CDCA	ND	0.04 ± 0.07	0.01 ± 0.02
UDCA	ND	0.1 ± 0.1	ND
CA	0.4 ± 0.5	0.3 ± 0.7	0.3 ± 0.2
ωMCA	ND	0.02 ± 0.07	ND
αMCA	0.3 ± 0.2	0.3 ± 0.6	0.2 ± 0.2
βMCA	0.9 ± 0.7	1.3 ± 2.7	0.8 ± 0.7
GCA	ND	0.1 ± 0.1	0.1 ± .08
TCDCA	3.3 ± 1	4 ± 2.1	2.5 ± 1.3
TUDC A	6.6 ± 1.4	6.6 ± 5.2	5.1 ± 1.02
TβMCA	50 ± 4.8	49.2 ± 7.5	50.5 ± 5.2
TCA	38 ± 3.5	38 ± 6.2	40.4 ± 5

Relative composition in bile acids given in percentage of total bile acid content. Species not detected with UPLC-MS experiment are shown as ND. The key is given in UPLC-MS material and methods.

Integration of correlations between bile acids and fecal flora

We further investigated the connections between fecal flora and intestinal bile acids using a correlation analysis based bipartite graphical modelling approach (see Materials and methods; Figure 5) used previously to investigate the effects of gut microbiome humanization in germfree mice (Martin *et al*, 2007a). In the current study, we are working with a superior

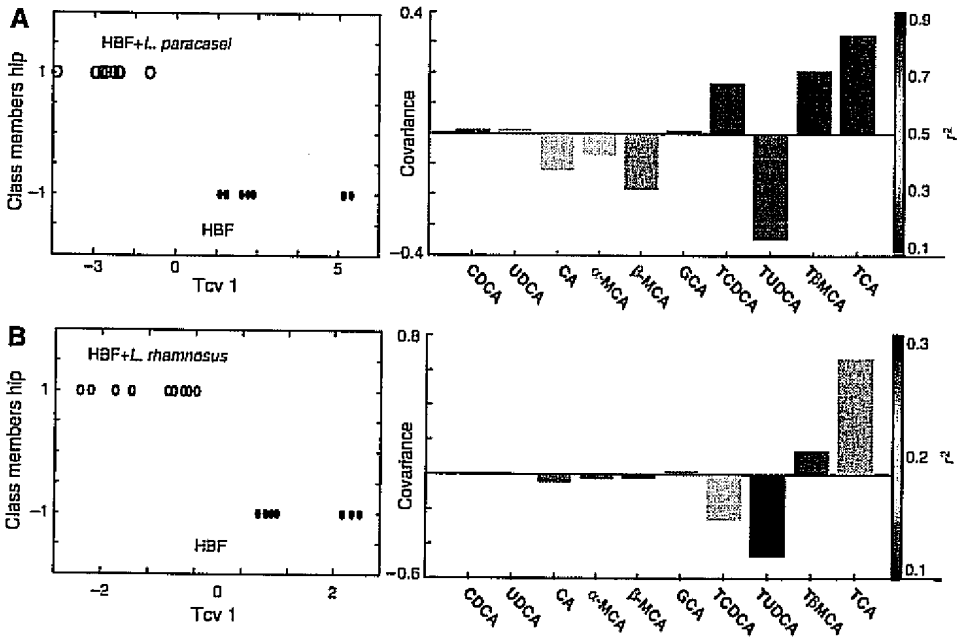

Figure 2 O-PLS-DA coefficient plots derived from the bile acid composition obtained by UPLC-MS analysis of ileal flushes, which indicate discrimination between HBF control mice (negative) and HBF mice treated with probiotics (positive), (**A**) *L. paracasei* and (**B**) *L. rhamnosus*. The color code corresponds to the correlation coefficients of the variables. One predictive and one orthogonal component were calculated; the respective Q^2_Y and R^2_X are (76.4, 52.2%) and (50.3, 51.2%).

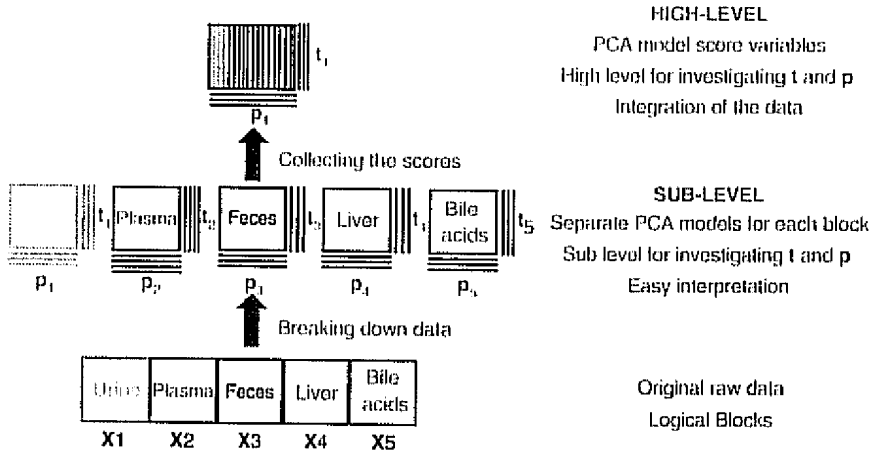

Figure 3 Schematic overview of H-PCA modelling: (Gunnarsson *et al*, 2003). In the sublevel, each block of data X*B* is modelled locally by a PCA model. Each block is summarized by one or more loading vectors $\mathbf{p}_b$ and score vectors $\mathbf{t}_p$ ('super variables'), which can be combined to form a new data matrix than can then be modelled using PCA, which generates the 'super scores' $\mathbf{t}_T$ and the 'super loadings' $\mathbf{p}_T$. All conventional PCA statistics and diagnostics are retained.

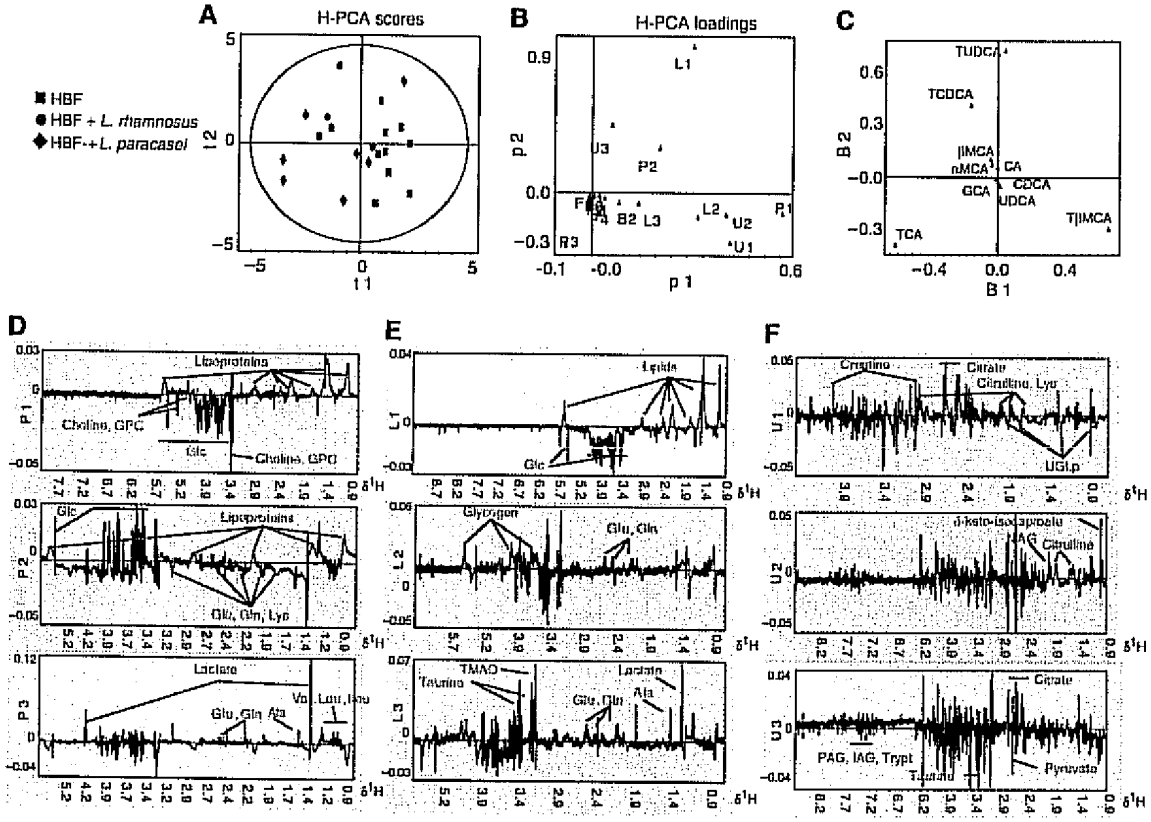

Figure 4 H-PCA scores (**A**) and loadings (**B**) plots for the two first components derived from scores of separate PCA constructed separately for the metabolic data from each individual biological matrix from HBF mice (■), HBF-*L. paracasei* mice (◆) and HBF-*L. rhamnosus* mice (○). These PCA models explained 94% (bile acid, **C**), 70% (plasma, **D**), 80% (liver, **E**), 41% (urine, **F**) and 61% (feces, data not shown) of the total variation in the data, respectively. The systematic variation from each of the block/compartment is summarized by its score vectors denoted P_i (plasma PCs 1–4), L_i (liver PCs 1–3), U_i (urine PCs 1–3), B_i (bile acid PCs 1–3) and F_i (fecal PCs 1–3), which can be combined to form a new data matrix than can then be modelled using PCA. The individual PCA loadings were color coded according to their contribution to the H-PCA model in red (principal component 1) and in blue (principal component 2). The model has been calculated from Pareto scaled data using two cross-validated PCs, R^2X=60%. Ala, alanine; see Figure 1.

model where all the major bacteria strains are identified, which was not possible when considering conventional microflora. Positive and negative correlations show the multicolinearity between bile acids and gut bacteria, whose concentrations are interdependent such as in the case of substrate–product biochemical reactions. Additional pixel maps of the correlation matrices are given to help interpretation in Supplementary Figure 1.

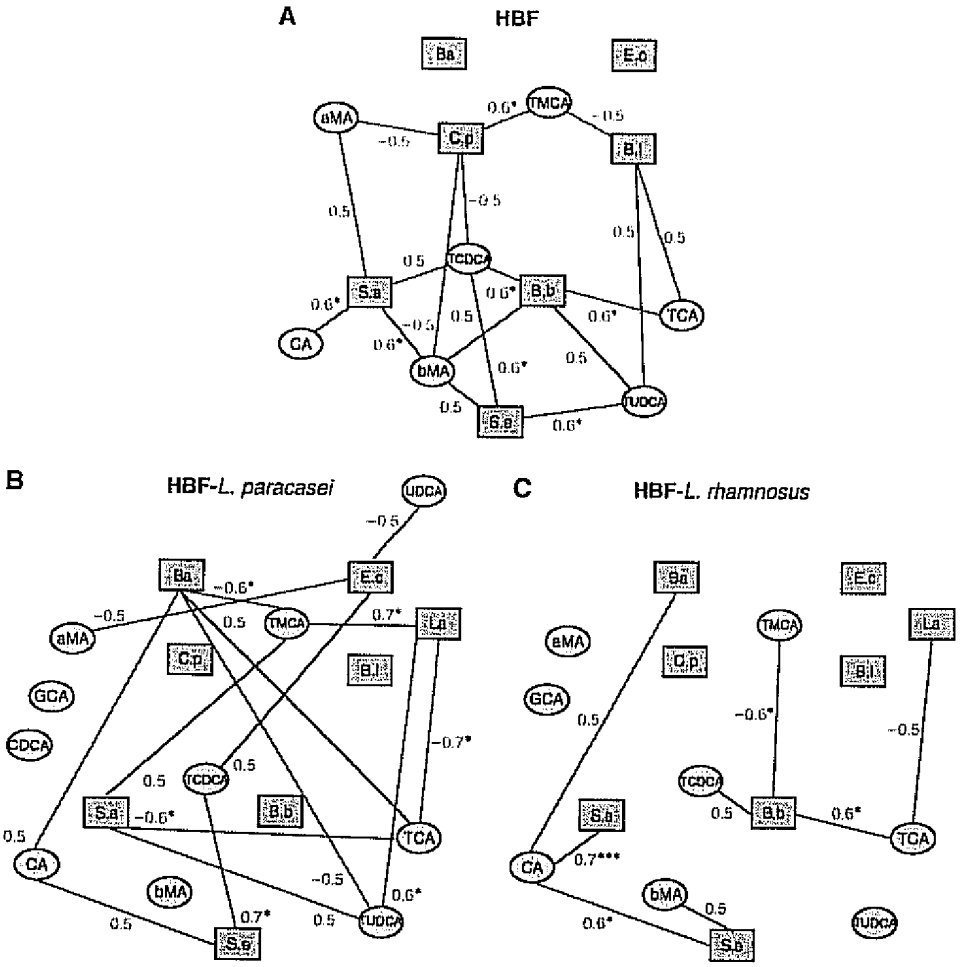

Figure 5 Integration of bile acid and fecal flora correlations. The bipartite graphs were derived from correlations between fecal flora and bile acids in each group: HBF mice (**A**), HBF mice supplemented with *L. paracasei* (**B**) or *L. rhamnosus* (**C**). The cut-off value of 0.5 was applied to the absolute value of the coefficient |*r*| for displaying the correlations between fecal flora and bile acids. Bile acids and fecal bacteria correspond to blue ellipse nodes and green rectangle nodes, respectively. Edges are coded according to correlation value: positive and negative correlations are respectively displayed in blue and in red. aMA, α-muricholic acid; Ba, *Bacteroides*; Bb, *B. breve*; Bl, *B. longum*; bMA, β-muricholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; Cp, *C. perfringens*; Ec, *E. coli*; GCA, glycocholic acid; La, *Lactobacillus* probiotics; Sa, *S. aureus*; Se, *S. epidermidis*; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TMCA, tauro-β-muricholic acid; TUDCA, tauroursocholic acid; UDCA, ursocholic acid. * and *** indicate a statistically significant correlation at 95 and 99.9% confidence levels, respectively.

Control HBF mice and HBF mice supplemented with probiotics show remarkably different bile acid/fecal flora correlation networks (Figure 5A–C), indicating that small modulations in the species composition of the microbiome can result in major functional ecological consequences. Network statistics reveal that microbiome/metabolome bipartite graphs from HBF mice supplemented with Lactobacilli show a totally different nodal structure (given for the cut-off value of 0.5). In particular, we observed that in the network obtained from control HBF mice, the most connected bacteria are Bifidobacteria, Staphylococci, and Clostridia, for which the variations are intrinsically correlated with the balance in tauro-conjugated bile acids (TCDCA, TβMCA, TCA, TUDCA) and unconjugated bile acids (CA, αMCA, βMCA). In particular, the potentially harmful opportunist *C. perfringens* shows functional correlation of opposite sign for TβMCA, TCDCA, αMCA and βMCA when compared to Bifidobacteria and *S. aureus*.

Network analysis for HBF mice supplemented with *L. paracasei* reveals that Lactobacilli supplementation resulted in decreasing the functional links between *Bifidobacteria* and bile acids, while new significant correlations were observed between bile acids, and *Bacteroides*, *S. aureus*, *S. epidermidis* and *L. paracasei*. Moreover, *E. coli* has several connections with UDCA, αMCA and TCDCA. Interestingly, *Bacteroides* shows functional correlations of opposite signs for TβMCA, TUDCA and TCA when compared to Lactobacilli and Staphylococci.

When HBF mice received *L. rhamnosus* probiotic, the microbiome/metabolome network shows a significant lower level of complexity (given for the cut-off value of 0.5). The balance within *B. breve*, *S. aureus* and *S. epidermidis* appears highly correlated to the composition in tauro-conjugated bile acids (TβMCA, TCA) and unconjugated bile acids (βMCA, CA).

Discussion

The cometabolic processes regulating mammalian systems and their coexisting gut microbiota are an essential evolutionary driver towards providing more refined control mechanisms on the host physiology (Pereira and Gibson, 2002; Pereira *et al*, 2003; Backhed *et al*, 2004; Holmes and Nicholson, 2005; Nicholson *et al*, 2005; Dumas *et al*, 2006; Martin *et al*, 2006, 2007a). In the present study, we demonstrate a significant association between the probiotic modulation of the gut microbiome and the metabolism of SCFAs, amino acids and methylamines, bile acids and plasma lipoproteins, and also an association with stimulated glycolysis, showing the remarkable diversity of symbiotic cometabolic connections.

Gut microbiotal links to host energy metabolism

We have recently described that HBF mice supplemented with *L. paracasei* were characterized by a high gut content of tauro-conjugated bile acids due to the inability of gut flora to deconjugate the bile acids, which resulted in high intestinal absorption of dietary lipids, accumulation of lipids in the liver and reduction of lipoprotein levels in plasma when compared to conventional animals (Martin *et al*, 2007a). Here, we show that probiotics supplementation of HBF mice resulted in a decrease in the plasma concentrations of VLDL and low-density lipoproteins (LDL) and increased triglyceride and GPC concentrations in plasma when compared to HBF controls (Figure 1B). In addition, the *Lactobacillus* supplementation resulted in decreased fecal excretion of bile acids (Figure 1C), that may be caused by accumulation of bile acids in *Lactobacillus* probiotics (Kurdi *et al*, 2000). These observation indicated probiotic-induced changes in the enterohepatic recirculation of bile acids, which were shown to lower cholesterol and systemic levels of blood lipids (Pereira and Gibson, 2002). Moreover, probiotic-specific modulation of the ileal concentrations of UDCA and CDCA (Figure 2; Table IV) may also contribute to modulation of the synthesis and secretion of VLDL into the blood (Lin *et al*, 1996; Watanabe *et al*, 2004).

Moreover, the main source of dietary lipids in animal chow is soybean oil, which is composed at 65% of long-chain polyunsaturated fatty acids. It is well known that *Lactobacillus* hydrolyzes soy oil to conjugated linoleic acid efficiently (Xu *et al*, 2005), which results in a reduction of plasma lipoprotein concentrations and hepatic cholesterol (Fukushima *et al*, 1996, 1997; Al-Othman, 2000) and in the inhibition of *S. aureus* growth (Das, 2002), as observed in the current study. Furthermore, probiotics supplementation was associated with significant reduction of acetate in the cecal content (Table II) and in a reduced hepatic acetate:propionate ratio, for which a serum lipids lowering effect has previously been described (Wong *et al*, 2006). Our results illustrate the fine relationship between a specific gut microbial population modulation and the host's lipid metabolism and that a probiotic intervention can provide refined control mechanisms on the host's physiology.

Furthermore, the molecular foundations of beneficial symbiotic host–bacteria relationships lie in the critical involvement of the microbiome in calorie recovery through further processing of dietary nutrients and indigestible fibers. Levels of leucine and isoleucine were reduced together with their keto-acid derivative (α-keto-isocaproate) in *L. paracasei*-supplemented mice. These observations suggest higher catabolism of branched-chain amino acids to produce acetyl-CoA and glucose *via* gluconeogenesis. Decreased levels of citrate in urine and plasma, but increased liver succinate levels may also indicate the shunt of the tricarboxylic acid cycle towards production of phosphoenolpyruvate for gluconeogenesis in *L. paracasei*-supplemented mice. Moreover, reduction of liver glycogen observed with *L. paracasei* supplementation is consistent with our other observations of generalized host mobilization of other metabolic fuels.

Probiotics induce specific microbiome–host transgenomic metabolic interactions

We investigated the relationship between probiotic-induced changes in gut microbes and bile acid cometabolism using bipartite graphs to display correlation patterns between fecal flora and bile acids (Figure 5). Correlation analysis derived from bile acid and fecal flora profiles offers a unique approach to capture subtle variations in bile acid composition that may be directly related to changes in gut microbial levels, and that may be induced by accumulation of bile acids in *Lactobacillus* probiotics for instance. Control HBF mice and HBF mice supplemented with probiotics show remarkably different bile acid/fecal flora correlation networks. The different bacterial strains of Bacteroides, Clostridia, Streptococci and Lactobacilli share similar abilities to deconjugate the hepatic tauro-conjugated bile acids (Midtvedt and Norman, 1967; Floch, 2002). In that regard, the overwhelming contrast between the balance of these bacteria on one hand, and conjugated bile acids (TCDCA, TβMCA, TCA, TUDCA) and unconjugated bile acids (CA, αMCA, βMCA) on the other hand, highlights the metabolic flexibility of the gut microbiota in response to probiotics supplementation. These different correlative patterns further characterize the microbial–mammalian transgenomic metabolic interactions, whereby probiotics-induced modulation of the gut microbial functional ecosystem results in different bile acid composition (Figure 2) and enterohepatic recirculation.

The relationship between specific gut microbial strains and bile acid cometabolism is well illustrated with the contrast between *Lactobacillus*, which shows resistance to bile salt toxicity (Corcoran *et al*, 2005), and *C. perfringens*, which is sensitive to the strong growth inhibitory effects of unconjugated bile acids and TCDCA (Kishinaka *et al*, 1994; Floch, 2002). In the absence of *Lactobacillus* supplementation, *C. perfringens* has anticorrelated connections with TCDCA, αMCA and βMCA, which highlights the strong inhibitory effects of these bile acids on *C. perfringens* growth. Interestingly, the probiotic supplementation was either associated with maintenance or decrease of the Clostridial population (Table I), while no functional correlations between *C. perfringens* and bile acids were observed. In that regard, these observations may indicate different nutritional competition leading to modulation of *C. perfringens* population and maintainance of the intestinal ecology. Consequently, inter-bacterial cooperation to transform bile acids is an important

factor that needs to be considered not only for the fine tuning of microbial balance but also to modulate dietary fat emulsification and absorption.

Gut-bacterial production of methylamines via choline metabolism

The elevation of methylamines (TMA, TMAO) in liver, choline and GPC in the plasma and decreased choline in feces from HBF mice supplemented with *L. rhamnosus* (Figure 1) are additional illustrations of the complex 'microbial–mammalian metabolic axis,' as the microbiota are involved in the synthesis and metabolism of these methylamines (al-Waiz *et al*, 1992). The first reaction of the methylamine pathway involves conversion of dietary choline into TMA by gut microbiota (Zeisel *et al*, 1983), which is then detoxified to TMAO in the liver *via* the flavine monooxygenase system (Smith *et al*, 1994; (Figure 6). *L. rhamnosus* supplementation contributes to higher absorption of free choline and may induce elevated production of methylamines by *Bacteroides* and *C. perfringens* (Allison and Macfarlane, 1989) through nutritional competition.

Interestingly, *L. paracasei* consumption may favor a different metabolic fate for choline through different bacterial reprocessing. Decreased fecal choline was associated with reduced concentrations of TMA and DMA in the liver, an increase in plasma GPC, but with no changes in liver TMAO and plasma choline after *L. paracasei* supplementation. These animals also showed a greater reduction in plasma lipoproteins when compared to other groups. Here, these observations may result from elevated bacterial consumption for cholesterol assimilation (Rasic, 1992) and phospholipid metabolism (Jenkins and Courtney, 2003; Taranto *et al*, 2003; Kankaanpaa *et al*, 2004). Thus, the reduced availability of choline to other bacterial strains may have led to lower production of methylamines and absorption of free choline into host metabolism.

Probiotic modulation of amino-acid metabolism

Investigation of the urine metabolic profiles showed significant increases in the concentrations of microbial cometabolites PAG, IAG and tryptamine in probiotic supplemented mice (Goodwin *et al*, 1994; Smith and Macfarlane, 1996). These metabolites are produced from amino acids, which after

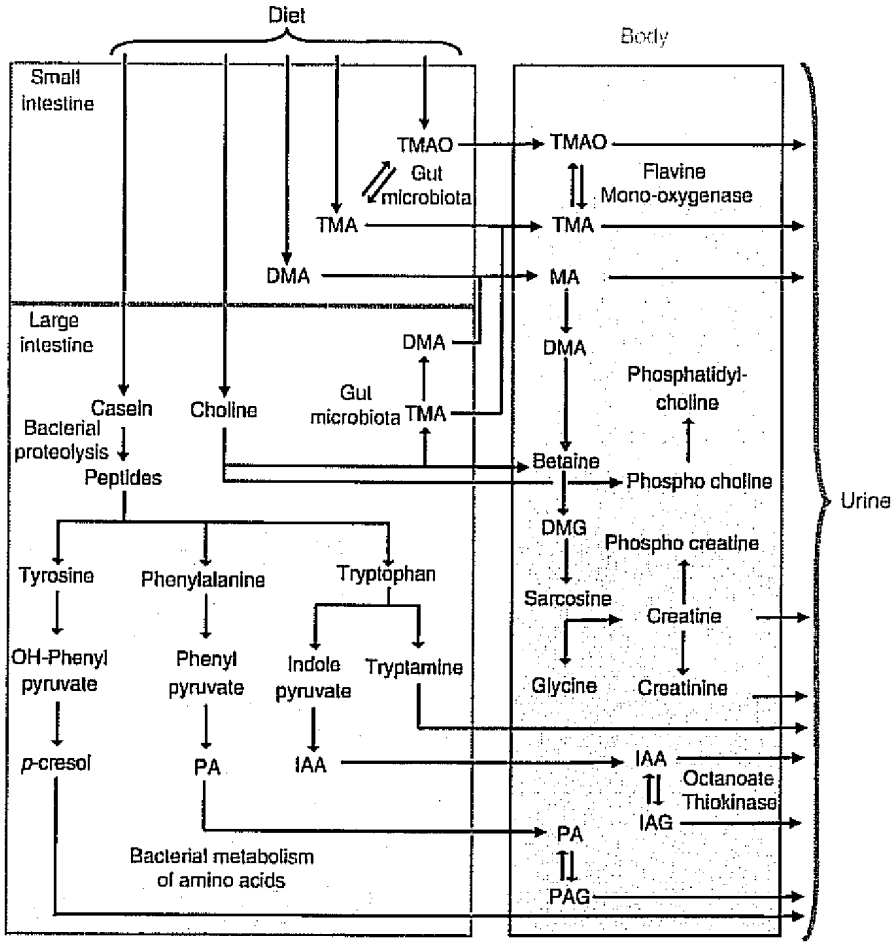

Figure 6 Gut-microbiota–mammalian cometabolism of methylamines and aromatic amino acids. DMG, dimethylglycine; IA, indoleacetate; MA, methylamine; PA, phenylacetate (see Figure 1).

depolymerization of dietary proteins (casein) by pancreatic endopeptidases and bacterial proteases and peptidases, become available for fermentation by the gut microflora (Smith and Macfarlane, 1996), as outlined in Figure 6. Metabolism of the aromatic amino acids phenylalanine, tyrosine and tryptophan generates phenylacetate (PA), *p*-cresol, indoleacetate (IA) and tryptamine, respectively (Donaldson, 1962; Smith and Macfarlane, 1996). PA and IA may be detoxified in the gut mucosa and the liver by glycine conjugation forming PAG and IAG prior to excretion *via* the urine (Donaldson, 1962; Smith and Macfarlane, 1996). The production of PA and IA has been restricted to a certain taxonomic group of gut bacteria, including *Bacteroides*, *Clostridia* and *E. coli*, which count among the dominant species in HBF colonized mice (Smith and Macfarlane, 1996; Xu *et al*, 2002). The increased urinary excretion of phenolic and indolic compounds reflects variations in gut microflora composition in relation to nutritional competition (Smith and Macfarlane, 1996; Nowak and Libudzisz, 2006). For instance, IA has been reported to inhibit the growth and survival of Lactobacilli, and specifically *L. paracasei* (Nowak and Libudzisz, 2006).

In addition, increased levels of lysine in feces from mice supplemented with probiotics is another example of bacterial contribution to the mammalian amino-acid homeostasis (Metges, 2000) and hepatic protein synthesis (Metges *et al*, 2006; Figure 1). Higher production of bacterial isobutyrate and isovalerate (Table II) suggests increased bacterial fermentation of leucine and valine that can also influence host energy metabolism (Macfarlane *et al*, 1992). Moreover, *L. paracasei* supplementation specifically induced higher urinary excretion of NAG and decreased *N*-acetylated metabolites in fecal extracts. A relationship between high casein diet and urinary excretion of NAG has been described previously (Hallson *et al*, 1997), which also suggested elevated bacterial proteolysis.

Altogether, our data suggest that the probiotic-induced increased proteolytic activities may reflect the basal metabolism of these *Lactobacillus* strains, in particular *L. paracasei* for which the proteolytic activities on casein medium are known to be elevated (Sasaki *et al*, 1995; Ikram and Mukhtar, 2006). Moreover, increased urinary NAG levels have been reported as a biomarker of increased tubular activity and tubular cell toxicity. Changes in bacterial fermentation of carbohydrates can lead to different ion absorption from the gut (Scholz-Ahrens *et al*, 2001), which may contribute to altered kidney metabolism and tubular activity. Therefore, the gut microbial contribution to the modulation of the NAG biomarker is certainly of potential toxicological assessment significance.

H-PCA modelled multicompartmental matrices related to lipid metabolism

H-PCA has been explored for the first time as a top-down systems approach to model and integrate metabolic profiles from diverse biological compartments. H-PCA modelling summarized clearly the intercorrelated changes induced by probiotic-treatment in plasma, urine and liver matrices and composition in bile acids (Figure 4). One benefit of the hierarchical approach lies in the much simplified simultaneous visualization of global system biochemical changes in multibiological matrices and improves interpretability. For instance, H-PCA resulted in a separation between the treated groups, which was not observed in separate PCA models. In addition, the H-PCA loadings plot gives the relative importance of the different blocks (biological matrices) in carrying diet-induced discriminant information. Moreover, the relationships between the descriptors in the simplified H-PCA space (H-PCA loadings plot) indicate correlations and anticorrelations between block variables, which may give insight into correlations between the metabolic variations in different biological matrices as exemplified here by metabolic changes in liver, plasma and urine and related to lipid metabolism. The H-PCA model also efficiently summarized the intercorrelated changes related to higher systemic glycolysis in plasma, urine and liver matrices, that is, reduced ketone body formation, anaerobic glycolysis, tricarboxylic cycle perturbation and amino-acid catabolism (Figure 4). Such observations might lead to better description of multiorgan metabolic perturbations (Figure 4B). This multicompartmental top-down approach offers a way forward to study the systemic biochemical profiles and regulation of function in whole organisms by analyzing simultaneously several metabolite pools from different biofluids and tissues. This approach also provides a new strategy for the quantitative and qualitative evaluation of different probiotic (or indeed any functional food or nutriceutical) interventions in relation to host biochemistry.

Conclusion

Significant associations between host metabolic phenotypes and a nutritionally modified gut-microbiota strongly supports the idea that changes across a whole range of metabolic pathways are the product of extended genome perturbations that can be oriented using probiotic supplementation, and which may play a role in host metabolic health. Bipartite network analysis highlights the metabolic flexibility of the gut microbiota, whereby bacterial strains communicate with each other to metabolize differently bile acids in a gut microbial ecosystem modulated with probiotics. In this case, probiotic consumption exerted a modification over the microbiome resulting in different hepatic influx and efflux of fatty acids in the liver, as observed with increased enterohepatic recycling of bile acids and dietary fats, lowered plasma lipoprotein levels and stimulated glycolysis. Probiotics also induced a different microbial proteolytic activity as well as modulation of bacterial metabolism of amino acids, methylamines and SCFAs. We showed the novel application of H-PCA as a means to study perturbation of metabolic profiles triggered by symbiotic microbiota at a 'global system' level by analyzing several metabolite pools simultaneously from different biofluids and tissues. These integrated system investigations demonstrate the potential of metabolic profiling as a top-down systems biology driver for investigating the mechanistic basis of probiotic action and the therapeutic surveillance of the gut microbial activity related to dietary supplementation of probiotics and their health consequences.

Materials and methods

Animal handling procedure

All animal studies were carried out under appropriate national guidelines at the Nestlé Research Center (Lausanne, Switzerland). The HBF is constituted of a total of seven bacterial strains, isolated from stool of a 20-day-old female baby that was naturally delivered and breast-fed, namely *E. coli*, *B. breve* and *B. longum*, *S. epidermidis* and *S. aureus*, *C. perfringens* and *Bacteroides distasonis*. Bacterial cell mixtures contain approximately 10^{10} cells/ml for each strain and were kept in frozen aliquots until use. *Lactobacillus paracasei* NCC2461 and *L. rhamnosus* NCC4007 probiotics were obtained from the Nestlé Culture Collection (Lausanne, Switzerland).

A total of 28 female germ-free mice (C3H strain), aged 6 weeks, received a single dose of HBF bacteria mixture and will be called HBF mice in the current manuscript. The experimental design is detailed in Supplementary Figure 2. The animals were fed with a standard pathogen-free rodent diet constituted of 50% cornstarch, 20% casein, 10% sucrose, 7% soybean oil, 5% cellulose, 0.25% choline bitartrate, 0.3% cystine and vitamin and mineral mixtures (Reeves *et al*, 1993) for 2 weeks. A control group of HBF mice ($n=10$) received a saline drink *ad libitum* containing Man, Rogosa and Sharpe (MRS) culture medium and was fed with a basal mix diet containing in composition 2.5% of a glucose–lactose mixture (1.25% each) for 2 additional weeks. Two groups of HBF mice were given a daily probiotic supplement, either *L. paracasei* (group A, $n=9$) or *L. rhamnosus* (group D, $n=9$), containing around 10^8 probiotic bacteria in MRS per day mixed with the saline solution *ad libitum* and were also fed with the basal mix diet.

Fecal pellets were first collected in the morning for microbiological analysis and frozen at −80°C for NMR spectroscopic analysis. Urine was then collected from animals prior to euthanasia and frozen at −80°C. Urine samples were not obtained for every animal, as some mice had an empty bladder at the time of killing (group A, $n=8$; group B, $n=6$; group C, $n=8$). Blood (400 µl) was collected into Li-heparin tubes and plasma was obtained after centrifugation and frozen at −80°C. The ileum and liver were dissected and snap-frozen. Ileal flush samples were obtained by rinsing the ileal lumen using a 1 ml sterile syringe containing a phosphate buffer solution (0.2 M Na_2HPO_4/0.04 M NaH_2PO_4, pH 7.4) for UPLC-MS analysis. Cecal content was collected upon animal autopsy, snap-frozen immediately and maintained at −80°C prior to analysis.

Microbial profiling of fecal contents

Immediately after collection, fecal pellets were homogenized in 0.5 ml Ringer solution (Oxoid, UK) supplemented with 0.05% (w/v) L-Cystein (HCl). The enumeration of specific microorganisms was performed after plating and incubation of different dilutions of the bacterial solution on selective and semiselective media, for example, *Bifidobacteria* on Eugom Tomato medium, *Lactobacillus* on MRS + antibiotic (phosphomycine, sulfamethoxazole, trimethoprim) medium, *C. perfringens* on NN-agar medium, *Enterobacteriaceae* on Drigalski medium and *Bacteroides* on Shaedler Neo Vanco medium. *Enterobacteriaceae* cultures were incubated at 37°C under aerobic conditions for 24 h, and other cultures were incubated under anaerobic conditions over a 48 h period.

Gas-chromatography on cecal content

An aliquot of cecal content was extracted with 4 ml buffer (0.1% (w/v) $HgCl_2$ and 1% (v/v) H_3PO_4 supplemented with 0.045 mg/ml 2,2-dimethylbutyric acid (as internal standard) per gram fresh weight. The resulting slurry was centrifuged for 30 min at 5000 *g* at 4°C. Fecal SCFAs were analyzed using a gas-chromatograph (HP 6890) equipped with a flame ionization detector and a DB-FFAP column (J&W Scientific, MSP Friedli & Co, Switzerland) of 30 m length, 530 µm diameter and 1 µm film thickness. The system was run with helium gas at an inlet constant pressure of 10 psi at 180°C. Each sample run was preceded with a cleaning injection of 1.2% formic acid. Samples were run at an initial temperature of 80°C for 1.2 min followed by heating to 145°C in 6.5 min, heating to 200°C in 0.55 min and an additional 0.5 min at 200°C. SCFAs were identified using external standards (acetate, propionate, iso-butyrate, *n*-butyrate, iso-valerate, *n*-valerate) and the concentration was calculated using the internal standard.

^{1}H NMR spectroscopic analysis

A volume of 100 µl of blood plasma was added to 450 µl of saline solution containing 10% D_2O, which was used as a spectrometer field frequency lock, into 5 mm NMR tubes. Urine samples were prepared by mixing 20 µl of samples with 30 µl of a phosphate buffer solution containing 90% D_2O and 0.25 mM 3-trimethylsilyl-1-[2,2,3,3-$^2H_4$] propionate (TSP), which was used as chemical shift reference (δ 0.0), into 1.7 mm NMR tubes. Fecal pellets were homogenized in 650 µl of a phosphate buffer solution containing 90% D_2O and 0.25 mM TSP. The fecal samples were sonicated for 30 min at 25°C and then centrifuged at 13 000 r.p.m. for 20 min to remove particulates. The supernatants were removed and centrifuged at 13 000 r.p.m. for 10 min. A 580 µl aliquot of the fecal supernatant was then pipetted into a 5 mm NMR tube for spectroscopic analysis. Portions of intact liver samples (~15 mg) were bathed in ice-cold 0.9% saline D_2O solution and packed into a zirconium oxide 4 mm outer diameter rotor.

^{1}H NMR spectra were acquired for each sample using a Bruker DRX 600 NMR spectrometer (Rheinstetten, Germany) operating at 600.11 MHz for ^{1}H. The instrument was equipped with a Bruker 5 mm TXI triple resonance probe maintained at 298 K for liquid samples and a standard Bruker high-resolution MAS probe under magic-angle-spinning conditions at a spin rate of 5000 Hz for intact tissues (Waters *et al*, 2000). Tissue samples were regulated at 283 K to minimize biochemical degradation.

One-dimensional (1D) ^{1}H NMR spectra were obtained from each sample using a standard solvent suppression pulse sequence (RD-90°-t1-90°-tm-90°-acquire FID) with t_m fixed at 100 ms and t_1 at 3 µs (Wang *et al*, 2005). Additional spin echo Carr-Purcell-Meiboom-Gill (CPMG) spectra were acquired for plasma and liver samples using the pulse sequence (RD-90°-(t-180°-t)$_n$-acquire FID), with a spin–spin relaxation delay, 2nτ, of 160 ms for plasma and 200 ms for tissue (Meiboom and Gill, 1958). The 90° pulse length was 9.0–12 µs. A total of 128 transients were collected into 32K data points with a recycle delay (RD) of 2 s. The assignment of the ^{1}H NMR spectral peaks to specific metabolites was achieved based on the literature (Nicholson *et al*, 1995; Fan, 1996), and confirmed by 2D COrrelation SpectroscopY (COSY) (Hurd, 1990) and TOtal Correlation SpectroscopY (TOCSY) (Bax and Davis, 1985). 2D NMR spectra were acquired on selected samples. Further assignment of the metabolites was also accomplished with the use of Statistical TOtal Correlation SpectroscopY (STOCSY) on 1D spectra (Cloarec *et al*, 2005a).

UPLC-MS methods

The Ultra Performance™ liquid chromatography of ileal flushes was performed on a ACQUITY UPLC system (Waters, Milford, MA, USA) equipped with a Tof™ LCT-Premier (Waters MS Technologies, Manchester, UK) for mass detection using the method we described previously (Martin *et al*, 2007a). The same conditions were applied for analysis of the following bile acid standards: cholic (CA), taurocholic (TCA), glycocholic (GCA), deoxycholic (DCA), taurodeoxycholic (TDCA), glycodeoxycholic (GDCA), chenodeoxycholic (CDCA), taurochenodeoxycholic (TCDCA), glycochenodeoxycholic (GCDCA), lithocholic (LCA), taurolithocholic (TLCA), glycolithocholic (GLCA), ursodeoxycholic (UDCA), tauroursodeoxycholic (TUDCA), glycoursodeoxycholic (GUDCA), hyocholic (HCA), α-muricholic (αMCA), β-muricholic (βMCA), ω-muricholic (ωMCA), tauro-β-muricholic (TβMCA) and tauro-α-muricholic (TαMCA) acids. We have previously given the molecular structure, the retention time and the mass to charge ratio (and not the molecular weight as published previously) of the observed ions. Unconjugated bile acids formed a formate adduct.

Data analysis

Microbial counts and SCFAs composition in the cecum were analyzed using a two-tailed Mann–Whitney test.

^{1}H NMR spectra were corrected manually for phase and baseline distortion and referenced to the chemical shift of the CH_3 resonance of alanine at δ 1.466 for plasma and liver samples, to the TSP resonance of alanine at δ 0.0 for urine and fecal samples using XwinNMR 3.5 (Bruker Biospin, Rheinstetten, Germany). The spectra were converted into 22K data points over the range of δ 0.2–10.0 using an in-house developed MATLAB routine. The regions containing the water resonance (δ 4.5–5.19), and for urine urea resonance (δ 4.5–6.2), were removed. Chemical shift intensities were normalized to the sum of all intensities within the specified range before chemometric analysis.

UPLC-MS data were processed using the Micromass MarkerLynx™ applications manager Version 4.0 (Waters Corp. Milford, USA). The peaks of bile acids were identified by comparing the *m/z* ratio and retention time to the set of standard bile acids measured under the same conditions. Data were noise-reduced in both of the UPLC and MS domains using MarkerLynx standard routines. Integration of the UPLC-MS bile acid peaks was performed using ApexTrack2™. Each peak integral was expressed as a ratio to the sum of integrals of the 21 measured bile acids.

The multivariate pattern recognition techniques used in this study were based on PCA (Wold *et al*, 1987), H-PCA (Westerhuis *et al*, 1998) and the orthogonal-projection to latent structure (O-PLS) (Trygg and Wold, 2003). PCA was carried out using the SIMCA-P 11 software (Umetrics, Umeå, Sweden) in order to detect the presence of inherent similarities between metabolic profiles. Both NMR and LC-MS variables were subjected to Pareto scaling, by dividing each variable by the square root of its standard deviation. Data were visualized by means of principal component scores and loadings plots. Each point in the scores plot represents an individual biochemical profile of a sample, whereas on the loadings plot each coordinate represents a single NMR spectral region or LC-MS retention time and mass to charge ratio (*m/z*). Because the scores and loadings plots are complementary, biochemical components responsible for the differences between samples detected in the scores plot can be extracted from the corresponding loadings plot.

O-PLS-DA was also carried out using the method developed by Trygg *et al* (Trygg and Wold, 2003) and implemented for NMR spectral data by Cloarec *et al* (2005b) to exclusively focus on the effects of probiotic supplementation. All O-PLS-DA models were constructed using one predictive and two orthogonal components using data scaled to unit variance (i.e. by dividing each variable by its standard deviation). Here, the test for the significance of the Pearson product–moment correlation coefficient was used to calculate the cut-off value of the correlation coefficients at the level of $P<0.05$. To test the validity of the model against over-fitting, the cross-validation parameter Q^2 was computed and the standard seven-fold cross validation method was used (Cloarec *et al*, 2005b). The interpretation of the model was achieved from correlation coefficient plots that incorporated a back-transformation method such that the coefficients resembled the original structure of the NMR spectral data (Cloarec *et al*, 2005b).

H-PCA methods have been proposed in the recent literature to improve the interpretability of multiple models (Westerhuis *et al*, 1998) generated from several blocks of descriptor variables measured on the same objects (Bergman *et al*, 1998; Lundstedt *et al*, 1998; Janne *et al*, 2001). The data are divided into well-defined logical blocks X1, …, *XB* (where the index B refers to each biosample type), according to an event, instrumentation or biological compartment. A consensus direction among all the blocks is sought. Here, logical blocks were defined according to the biological nature of the samples obtained from the same animals (Figure 3). Hence, five independent blocks of variables were comprised of NMR data from plasma, liver, urine and fecal extracts as well as bile acids composition obtained by UPLC-MS. PCA was performed on each data matrix individually, and block loadings $\mathbf{p}_b$ and scores $\mathbf{t}_b$ were generated for each block (Figure 3). All block scores $\mathbf{t}_b$ were then combined into a super block T. The PCA (H-PCA) was then applied to the super block T to generate the super scores $\mathbf{t}_T$ and the super loadings $\mathbf{p}_T$. The super scores $\mathbf{t}_T$ gives the relationships between the observations and all the sources of variations. The super loadings $\mathbf{p}_T$ gives the relative importance of the different blocks XB for each principal component as well as information on the relationship/covariance between the different variables in the different compartments.

Bipartite graph representation of bile acid and fecal flora profiles

The bipartite graph (Rgraphsviz) package from *R* was used to display the correlation matrix derived from bile acid and fecal flora profiles to assess the probiotic-induced changes to the microbial-mammalian transgenomic interactions (Figure 5). Pearson's correlation coefficients were computed between bile acid variables and fecal flora variables from the same mice and a cut-off value of 0.5 was applied to the absolute value of the coefficient $|r|$ so that the bipartite graph only represents the correlations between the two types of nodes (fecal flora and bile acids) above the cut-off (Martin *et al*, 2007a). The sign of the initial correlation was then color coded (red: negative, blue: positive), and the correlation value displayed on the bipartite graph. In that context, presence of edges between two specific nodes (one of each type) reveals a functional correlation (above the cut-off) between these entities.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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EXHIBIT E

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The lower genital tract microbiota in relation to cytokine-, SLPI- and endotoxin levels: application of checkerboard DNA-DNA hybridization (CDH)

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Nikolaitchouk N, Andersch B, Falsen E, Strömbeck L, Mattsby-Baltzer I. The lower genital tract microbiota in relation to cytokine-, SLPI- and endotoxin levels: application of checkerboard DNA-DNA hybridization (CDH). APMIS 2008;116:263–77.

In the present study the lower genital tract microbiota in asymptomatic fertile women (n=34) was identified and quantified by culturing vaginal secretions. Also, vaginal and cervical samples were analyzed by a semiquantitative checkerboard DNA-DNA hybridization technique (CDH) based on genomic probes prepared from 13 bacterial species (*Bacteroides ureolyticus, Escherichia coli, Fusobacterium nucleatum, Gardnerella vaginalis, Mobiluncus curtisii* ss *curtisii, Prevotella bivia, Prevotella disiens, Prevotella melaninogenica, Atopobium vaginae, Lactobacillus iners, Staphylococcus aureus* ss *aureus, Streptococcus anginosus*, and *Streptococcus agalactiae*). The bacterial species found by either culture or CDH were correlated with proinflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8), secretory leukocyte protease inhibitor (SLPI), and endotoxin in the cervicovaginal samples. Grading the women into healthy, intermediate, or bacterial vaginosis (BV) as based on Gram staining of vaginal smears, the viable counts of lactobacilli (*L. gasseri*) and of streptococci-staphylococci combined were highest in the intermediate group. In BV, particularly the high concentrations of *Actinomyces urogenitalis, Atopobium vaginae*, and *Peptoniphilus harei* were noted ($\geq 10^{11}$ per ml). The total viable counts correlated with both cervical IL-1α and IL-1β. A strong negative correlation was observed between *L. iners* and total viable counts, *G. vaginalis*, or cervical IL-1α, while it correlated positively with SLPI. Analysis of vaginal and cervical samples from 26 out of the 34 women by CDH showed that anaerobic bacteria were more frequently detected by CDH compared to culture. By this method, *A. vaginae* correlated with *G. vaginalis*, and *L. iners* with *S. aureus*. With regard to cytokines, *B. ureolyticus* correlated with both cervical and vaginal IL-1α as well as with cervical IL-8, while *F. nucleatum, S. agalactiae, S. anginosus*, or *S. aureus* correlated with vaginal IL-1α. Furthermore, all Gram-negative bacteria taken together, as measured by CDH, correlated with vaginal endotoxin and inversely with vaginal SLPI. The significance of the results is discussed. In summary, mapping of the identity and quantity of vaginal bacterial species and their association with locally produced host innate immune factors will help in defining various types of abnormal vaginal microbiota, developing new ways of assessing the risk of ascending subclinical infections, and in treating them. CDH appears to be a suitable tool for future analyses of large numbers of clinical samples with an extended number of bacterial probes.

Key words: Bacterial vaginosis; *Atopobium vaginae*; cytokines; checkerboard DNA-DNA hybridization.

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Bacterial vaginosis (BV) is one of the most common disorders in gynecologic, obstetric, sexually transmitted disease practices, and health care control clinics. The frequency of BV has been shown to vary between 7–30% depending on the patient group studied. This vaginal syndrome is a condition where the normal *Lactobacillus*-predominant microbiota is replaced by an overgrowth of anaerobic bacteria. The most frequent bacteria isolated from women with BV include *Gardnerella vaginalis, Mobiluncus, Bacteroides, Prevotella* species, *Mycoplasma hominis, Peptoniphilus anaerobius* and other Gram-positive anaerobic cocci (1, 2). The underlying cause of BV is not known. It has been suggested that in some women hormonal changes during the first part of the menstrual cycle could have a role in the pathogenesis of BV (3). Sexual behavior may be important in the development of BV (4, 5).

BV is associated with an increased risk of gynecologic complications, including pelvic inflammatory disease, postoperative and postabortal infection of the upper genital tract, cervicitis, and possibly cervical intraepithelial neoplasia (6–10). In fact, some *P. bivia* strains have been shown to express a quite strong intracellular invasion capacity of cervix epithelial cells, which may have consequences for their ability to spread to the upper genital tract (11). Several population studies have demonstrated that BV is associated with an increased risk of HIV acquisition and transmission (12–14). The obstetric risk of BV includes spontaneous early pregnancy loss, spontaneous preterm premature rupture of membranes, spontaneous preterm labor, spontaneous preterm delivery, chorioamnionitis and postpartum endometritis (15–20). Vaginal colonization of anaerobes, including *Prevotella bivia, Peptostreptococcus anaerobius, Fusobacterium nucleatum, Ureaplasma urealyticum, Mycoplasma hominis* and *Mobiluncus* species, has been strongly associated with preterm labor and preterm birth (17). Since many BV-associated bacteria have been found within the amnion and fetal membranes of women delivering prematurely (15, 21), this provides strong indirect evidence that microbial invasion of the amniotic cavity results primarily from the ascent of microbes from the vagina.

The diagnosis of BV is based on clinical symptoms (Amsel's criteria) or Gram-stained vaginal smears (Nugent's score). However, it has also been shown that there are intermediate conditions according to Nugent scoring which may constitute a risk factor in pregnancy (16, 22). Thus, more information regarding the appearance and numbers of various bacterial species in the normal and disturbed vaginal microbiota and their relation to antimicrobial and inflammatory host defense factors is needed in order to define abnormal vaginal microbiota and identify risk factors for an ascending infection.

BV or an abnormal vaginal microbiota results in an inflammatory response characterized by an increased release of cytokines such as IL-1α/β and IL-8 in the lower genital tract (23–25). This release of cytokines is not necessarily accompanied by an inflammatory cellular response. In fact, BV is characterized by the absence of clinical inflammatory signs. In addition to cytokines, antimicrobial factors such as defensins and lactoferrin may contribute to the host response in BV (26). Vaginal secretory leukoprotease inhibitor (SLPI), which is a serine protease inhibitor expressing antibacterial-, antiviral- and antifungal activities, was shown to be reduced in women with BV (27–33). SLPI is produced by epithelial cells of mucosal surfaces, macrophages and neutrophils (34, 35). As SLPI has been shown to inhibit HIV-1 infection *in vitro* (36) it has been suggested that reduced levels of SLPI during a genital tract infection (BV, sexually transmitted diseases) may also increase susceptibility to contracting HIV-1 infection (37).

The aim of the present study was to analyze the lower genital tract microbiota in healthy fertile women by identification and quantification of all morphologically different colonies appearing upon culturing vaginal secretion. Also, a semiquantitative checkerboard DNA-DNA hybridization technique (13 chosen bacterial species), CDH, not requiring prior culture was applied and compared with the culture results (38). The concentration of various bacterial species as analyzed with both methods was related to endotoxin, proinflammatory mediators (IL-1, IL-6 and IL-8), and SLPI in vaginal secretion and cervical mucus.

MATERIALS AND METHODS

Study group

The study group consisted of 37 healthy non-preg-

nant women aged 22–51 years (mean (SD)=39 (8) years). All were consecutive outpatients who for gynecologic health care control or contraceptive advice had attended the Department of Obstetrics and Gynecology, Östra Hospital, Gothenburg, Sweden. In the present study, oral consent had been obtained and the same physician examined each patient. The examination included vaginal speculum exploration and bimanual palpation of uterus and adnexa. All women had a regular menstrual cycle. None of them had received any antibiotics or vaginal medication during the previous 3 months, or had any gynecologic diseases or complaints. The pelvic examination for each of these women was normal. Fourteen women were taking oral contraceptives, 19 women were nonusers of contraception, and 4 women had an intrauterine device.

Specimens were obtained during speculum examination of the vagina before any other vaginal examination. Care was taken to avoid contact with the external genitalia and other sources of contamination. Sterile cotton swabs (SARSTEDT, Sweden) were used for taking material from the posterior vaginal fornix for Gram-staining smears, culture, CDH, SLPI- and cytokine analyses. Cervical secretion was obtained by cytobrush (Medscand Medical, Sweden), which was inserted into the cervical channel and turned around. The cytobrush was used for CDH-, SLPI-, and cytokine analyses. Two separate samples were collected for culture of bacteria and CDH. The first cotton swab was designated for bacteriological processing and was placed in Amies transport media (Sarstedt AB, Sweden). The second swab and the cytobrush were placed in the separate sterile centrifuge tubes for CDH. The specimens were transported to the research laboratory within 2 h and the culture procedure was started immediately. The swab and the cytobrush were submerged in 1 ml of sterile distilled water and shaken for 1 h at 4°C followed by vortexing. The suspension was centrifuged at 12,000 *g* for 20 min at 4°C (Hettich Zentrifugen, GmbH, Germany). The supernatant was transferred to another tube for further analysis of SLPI and cytokines. Half a ml of sterile super-Q water was added to the pellet. Both pellets and supernatants were stored frozen at −70°C. Vaginal specimens from 34 of the 37 women were collected for quantitative culture, and within this group 26 random samples of each of the vaginal and cervical secretions were used for CDH analysis.

Vaginal smears were prepared and Gram stained using safranin. Each slide was scored as described by Nugent et al. (39). A score of 3 was considered to denote healthy microbiota, more than 3 but less than 7 intermediate microbiota, and 7-BV. Accordingly, the study population of 37 women was divided into 3 groups: normal grade (n=20), intermediate grade (n=10), and BV (n=7). Within the CDH analysis group the corresponding numbers of patients were 11, 9, and 6, respectively.

Identification and quantification of bacteria

The cotton swabs containing approximately 100 µl of vaginal secretion were placed into 1 ml oxygen-reduced 0.06 M NaCl sterile solution (40) and mixed vigorously on a vortex mixer for 3–5 min until the sample was completely dispersed. 10 serial 10-fold dilutions were prepared. Aliquots (10 µl) of all the dilutions were plated onto Columbia agar (Difco) supplemented with 5% horse blood, MRS agar (The Oxoid Manual, 6th Edition 1990), and chocolate agar. Sets of plates were incubated aerobically, in 5% CO_2, and anaerobically (Whitley Anaerobic Cabinet, Don Whitley Scientific Ltd., Shipley, West Yorkshire, England) with 10% H_2, 5% CO_2 and 85% N_2 gas mixture for 72 h at 37°C.

All colony types were counted, isolated, and characterized by morphologic features, hemolysis pattern, and Gram stain morphologic features. When the same organism was recovered from different plates, the highest count was used for determination of the final concentration. Concentrations of bacteria were expressed as cfu/ml of vaginal secretion (cotton swab). The isolated bacteria were cultured for comparative study on different media and conditions. Biochemically, identification was made using the API Rapid ID32STREP, Rapid ID32A, ID32STAPH, ID32E, API ZYM, API Coryne, API50CHL, ID32C according to the manufacturer's instructions (API BioMérieux, France). These systems identified *Staphylococcus*, *Streptococcus* and related organisms, coryneform bacteria, *Enterobacteriaceae* and other non-fastidious Gram-negative rods, the genus *Lactobacillus*, and yeasts. Polyacrylamide gel electrophoresis (PAGE) analysis of whole-cell proteins was performed (41). For densitometric analysis, normalization and interpretation of protein patterns, the GelCompar 4.1 software package (Applied Maths Gent, Belgium) was used. Anaerobic bacteria were furthermore subjected to fatty acid analysis using the MIDI specifications. Some of the difficult bacterial isolates were identified by DNA sequencing. The 16S rRNA genes of the isolates were amplified by PCR and directly sequenced using the Big dye terminator cycle sequencing kit (Applied Biosystems Foster City, USA) and an automatic DNA sequencer (model 310, Applied Biosystems).

CDH

Bacterial strains were cultured for the preparation of DNA templates (Table 1). Whole genomic DNA probes were prepared from 13 bacterial species associated with the vaginal microbiota [*Atopobium vaginae* (A.v), *Bacteroides ureolyticus* (B.u), *Escherichia coli* (E.c), *Fusobacterium nucleatum* (F.n), *Prevotella bivia* (P.b), *Prevotella disiens* (P.d), *Prevotella melaninogenica* (P.m), *Gardnerella vaginalis* (G.v), *Mobiluncus curtisii* ss *curtisii* (M.c), *Staphylococcus aureus* ss *aureus* (S.a), *Streptococcus anginosus* (Str.an), *Streptococcus agalactiae* (Str.ag), *Lactobacillus iners* (L.i)]. The selection was partly based on the bacterial

TABLE 1. *Bacterial strains used for the development of DNA templates*

Species	CCUG[a]	ATCC[b]
Bacteroides ureolyticus	44020B T	
Escherichia coli	29300 T	11775
Fusobacterium nucleatum	32989 T	25586
Prevotella bivia	9557 T	29303
Prevotella disiens	9558 T	29426
Prevotella melaninogenica	4944 T	25845
Gardnerella vaginalis	3717 T	14018
Mobiluncus curtisii ss *curtisii*	21018 T	35241
Staphylococcus aureus ss *aureus*	1800 T	12600
Streptococcus agalactiae	4208 T	13813
Streptococcus anginosus	27298 T	12395
Atopobium vaginae	43049	-
Lactobacillus iners	28746 T	-

[a] Culture Collection, University of Gothenburg.
[b] American Type Culture Collection.

TABLE 2. *Comparison of the concentration of five bacterial strains in five vaginal samples by culturing and CDH analysis*

	Culturing CFU/ml	CDH score*
L. iners	1.6×10^9	5 ($>10^6$)
A. vaginae	1.5×10^9	5 ($>10^6$)
G. vaginalis	8.4×10^{10}	5 ($>10^6$)
S. aureus	1.0×10^4	2 ($=10^5$)
S. agalactiae	1.0×10^4	1 ($<10^5$)

* Score, see text.

species identified in the 37 women (Table 3). The strains were cultured on Columbia agar supplemented with 5% horse blood and chocolate agar plates at 37°C under aerobic and anaerobic conditions for 24–72 h.

The bacteria were harvested from plates and dispersed in Tris-EDTA buffer (10 mM Tris HCl, 1.0 mM EDTA, pH 7.6) (TE). The bacterial suspension was centrifuged at 10 000 *g* for 10 min. and the pellet was resuspended in TE buffer. A series of two-fold dilutions was made and OD at 610 nm was measured in four to five different dilutions. The number of bacteria was estimated using a Bürker chamber. A standard curve was plotted for each bacterial strain. A bacterial stock solution was made for each strain with a concentration of 2×10^8 bacteria/ml. This solution was used for making standard bacterial mixtures at a concentration of 10^5 and 10^6 bacteria/ml in TE buffer/0.5 M NaOH (1:1 v/v) for each species. These mixtures were used in the DNA-DNA hybridization.

DNA preparation and labeling. Whole genomic bacterial DNA was prepared according to the Current Protocols of Molecular Biology (John Wiley & Sons, Inc., 2000) with some modifications by Smith et al. (42). DNA was labeled with digoxigenin using "DIG-High Prime" (Roche, Germany). The labeling procedure was performed according to the manufacturer's protocol. The probes were stored at +4°C until used.

Hybridization. The method was a modified version of the one described by Socransky et al. for dental plaque samples (38). The modifications consisted of a high stringency buffer and the following steps for the antibody reaction(43).

The chemiluminescence signals were obtained by exposure of the membranes to an X-ray film. A semiquantitative estimation was based on visual comparison with the bacteria mixture containing 10^5 and 10^6 of each species per ml according to Papapanou et al. (44). The quality of the DIG-labeled probes was tested and standardized by running different concentrations of DNA probes against the homologous bacterial strain and the pool. The probes were found to be specific and allowed a semiquantitative estimation of bacteria (Fig. 1a & b). A weak cross-reaction between *P. bivia* and *P. disiens*, and between *S. galactiae* probe and *S. anginosus*, was noted.

Thus, 0=no reaction, 1=fewer than 10^5 bacteria, 2=10^5, 3=more than 10^5 but fewer than 10^6, 4=10^6, and 5=more than 10^6. Each membrane contained positive (bacterial standards) and negative (without DNA) control lanes.

Analysis of vaginal samples from five women by both culture and CDH with respect to five bacterial species showed that the semiquantitative estimation by CDH agreed with the culture data regarding concentrations (Table 2).

Cytokine and SLPI assays

The levels of IL-1α, IL-1β, IL-6, and IL-8 in vaginal and cervical secretions were quantified by enzyme-linked immunosorbent assays (ELISA) based on matched anti-human IL-1α, IL-1β, IL-6, and IL-8 antibody pairs (R&D Systems Europe Ltd., UK). SLPI was based on a monoclonal antibody and biotinylated polyclonal antibodies directed against human SLPI. Recombinant SLPI was used as standard (R&D Systems, Abingdon, UK). Optical density measurements were performed using the Emaxprecision microplate reader (Molecular Devices Corporation, CA, USA). All samples were run in duplicate diluted 1:5, 1:20 and 1:100. The inter-assay variation

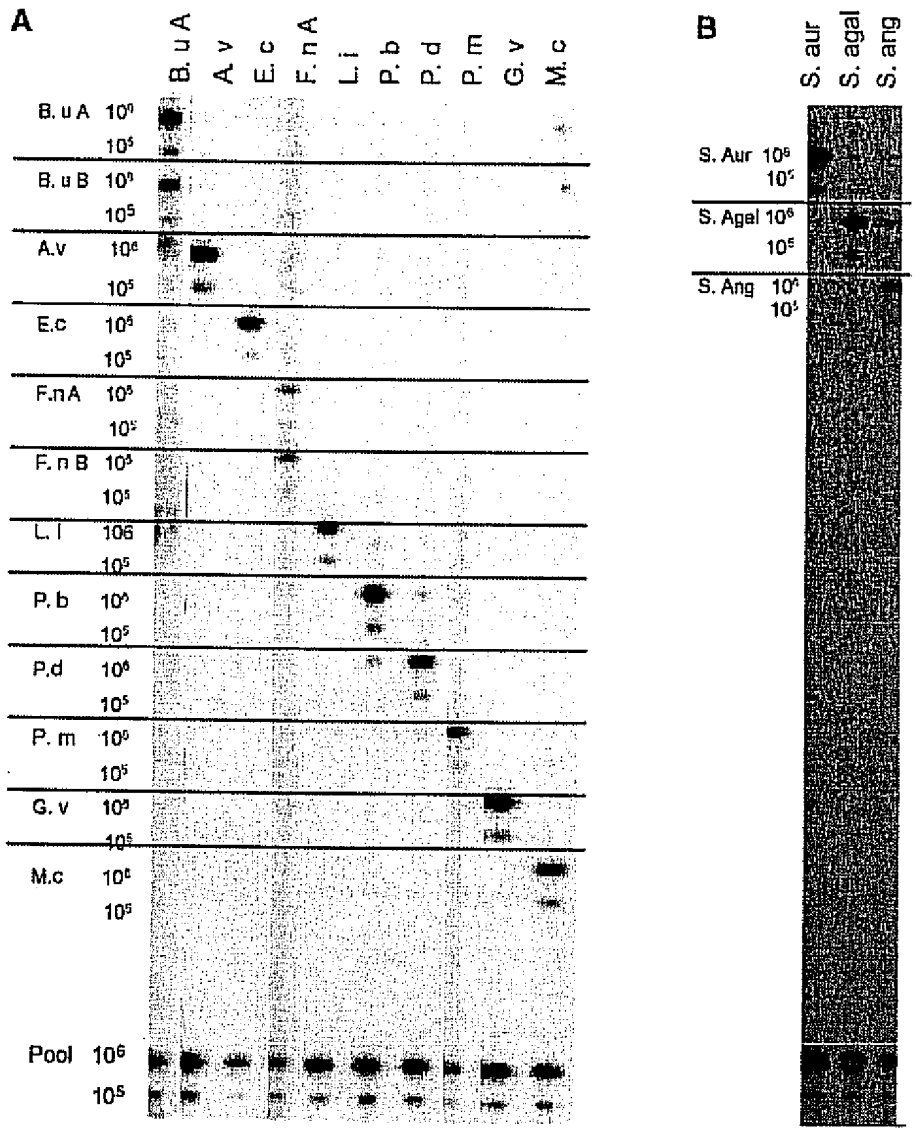

Fig. 1 A & B. The specificity of the labeled probes was tested with homologous bacterial strains at concentrations of 10^5 and 10^6 bacteria/ml. The reaction intensity against two heterologous bacterial strains (*B.u* B and *F.n* B) is also shown. The pool contained all species included in the analysis (probes added to the wells in 3–25 µl).

was calculated at < 10%, based on analysis of several samples on three separate occasions.

Endotoxin assay

The endotoxin content of the vaginal and cervical secretions was determined by endpoint Limulus amebocyte lysate test, Endochrome (Charles River Endosafe, SC, USA). The analysis was performed according to the manufacturer's manual. All samples were diluted 1:100 and run in duplicate. The limit for detection was 0.6 EU/ml (corresponding to 60 pg/ml) with respect to dilution of the sample.

Statistics

Spearman's rank correlation test and Kruskal-Wallis with Dunn's multiple comparison tests were performed.

RESULTS

Culture

Bacterial species in vaginal secretions. The numbers of bacterial species found in vaginal

TABLE 3. *All bacterial species found in vaginal fluid of 37 women. Range of the concentration (CFU/ml) and percentage of the total number of bacteria are listed as well as the number of women carrying the species*

Species	cfu/ml, range	% total cfu per sample, range	Number of women
Actinomyces neuii ss *neuii*	9×10^4–5.5×10^7	<0.01–0.1	3
Actinomyces urogenitalis	10^{12}	31.3	1
Aerococcus christensenii	7.6×10^8	0.8	1
Anaerococcus prevotii	ND		1
Atopobium vaginae	1.15×10^{10}–8×10^{11}	2.3–34.8	3
Bacteroides ureolyticus	ND		1
Brevibacterium	10^3	<0.01	1
Candida albicans	1.5×10^3–3×10^7	<0.01–23.1	6
Candida glabrata	3.7×10^8	0.5	1
Corynebacterium amycolatum	10^3	<0.01	1
Corynebacterium coyleae	2×10^3	<0.01	1
Corynebacterium minutissimum	10^3	<0.01	1
Corynebacterium seminale	10^5	0.01	1
Enterococcus faecalis	5×10^3–10^{10}	<0.01–93.8	3
Escherichia coli	1.8×10^6–6×10^8	<0.01–5.6	2
Fusobacterium	6×10^7	0.6	1
Gardnerella vaginalis	9×10^4–2×10^{12}	0.01–100	14
Lactobacillus coleohominis	2.25×10^8–4×10^9	0.4–0.8	2
Lactobacillus crispatus	10^7–5×10^{11}	12.8–100	8
Lactobacillus gasseri	10^3–10^{11}	<0.01–100	14
Lactobacillus iners	9×10^5–5×10^{10}	13.3–100	12
Lactobacillus jensenii	10^6–1.3×10^{11}	<0.01–86.7	4
Lactobacillus plantarum	10^4	<0.01	1
Lactobacillus reuteri	5×10^4	<0.01	2
Lactobacillus rhamnosus	1.3×10^6–1.2×10^7	<0.01–0.03	2
Lactobacillus vaginalis	10^4–1.2×10^7	<0.01	4
Micromonaslike	5×10^8	0.5	1
Mobiluncus mulieris	10^8	0.02	1
Peptoniphilus asaccharolyticus	2.6×10^5	<0.01	1
Peptoniphilus harei	2×10^{11}	6.3	1
Peptostreptococcus-like	8.5×10^4	2.1	1
Prevotella bucalis	1.3×10^8	0.1	1
Prevotella disiens	2×10^6	<0.01	1
Staphylococcus aureus ss *aureus*	10^3–2×10^3	<0.01	2
Staphylococcus epidermidis	10^3–10^5	<0.01	4
Staphylococcus haemolyticus	10^3	<0.01	1
Staphylococcus hominis ss hominis	2×10^3	<0.01	1
Staphylococcus warneri	10^4–6×10^4	<0.01	2
Streptococcus agalactiae	10^3–2.8×10^9	<0.01–97.9	6
Streptococcus anginosus	5.5×10^3–2×10^9	<001–1.8	6
Streptococcus intermedius	10^7	0.4	1
Streptococcus salivarius	6×10^4	<0.01	1

ND=not determined.

secretions of the 37 women are listed in Table 3. A total of 19 genera were detected. In addition, *C. albicans* and *C. glabrata* were isolated. The total number of bacteria (CFU) per ml of secretion ranged from 10^6 to 10^{12}, of which the highest concentrations, i.e. 10^{12}, were found for *A. urogenitalis* and *G. vaginalis*. Several *Lactobacillus* species as well as *P. harei* and *A. vaginae* were found in concentrations just below this limit. The most frequent species were *G. vaginalis*, *L. gasseri*, *L. iners*, and *L. crispatus*. Subsequently, the four most common *Lactobacillus* species were the three above mentioned in addition to *L. jensenii* (Table 3). Although *L.*

vaginalis was as frequent as *L. jensenii* it constituted less than 0.01% of the total concentration of bacteria.

Number of bacteria in relation to Nugent scoring, and other bacteria. The median numbers of bacteria were found in all three groups divided according to Nugent score (1.6×10^9, 3.0×10^{10}, and 5.1×10^{11} CFU/ml) (Fig. 2A). A correlation was found between total bacterial count and Nugent score ($r=0.3962$, $P=0.0204$).

The median values of the total number of lactobacilli per ml of vaginal secretion were 8.5×10^8 in the normal group, 1.3×10^{10} in the intermediate group, and $< 10^3$ in BV (Fig. 2B).

Streptococci (*S. anginosus, S. agalactiae, S. intermedius*, and *S. salivarius*), and staphylococci (*S. aureus, S. warneri, S. epidermidis, S. hominis, S. haemolyticus*) together occurred with the highest frequency in the intermediate group (80%) (Fig. 2C). Also, the intermediate group differed significantly from the normal group.

The median number of *G. vaginalis* was less than 1×10^3 CFU per ml in the normal group, 1×10^7 in the intemediate group, and 5×10^{11} in BV (Fig. 2D). Only the BV group differed significantly from the normal group ($P<0.001$). A strong correlation was found between *G. vaginalis* and Nugent score ($r=0.6808$, $P<0.0001$).

With regard to an association of *G. vaginalis* with other bacterial species, a strong negative correlation was seen between *G. vaginalis* and *L. iners*, and a weaker correlation for *L. crispatus* (Table 4). Despite being the most frequent *Lactobacillus* species, no correlation was found between *L. gasseri* and *G. vaginalis*. In fact, *L.*

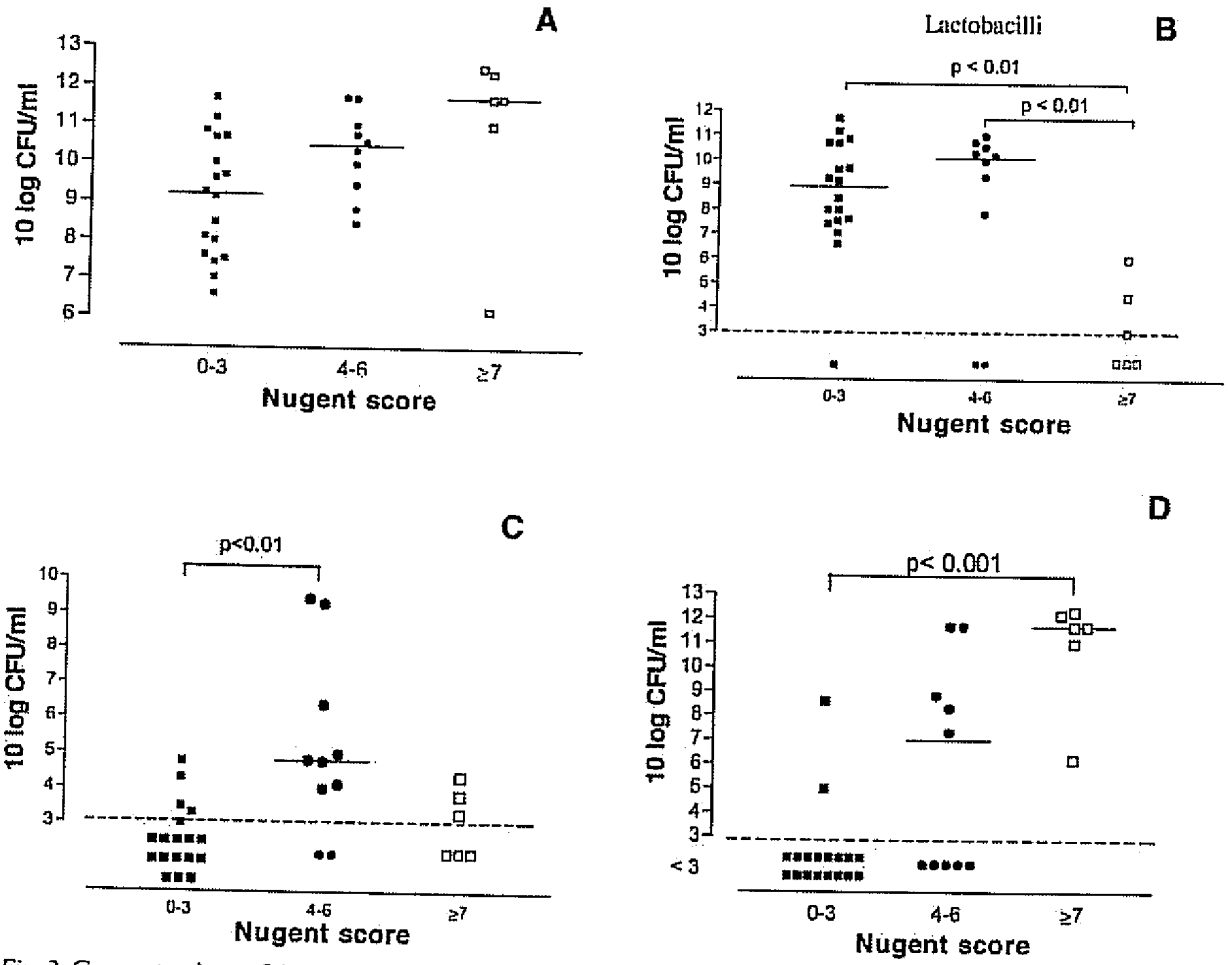

Fig. 2. Concentrations of A) total bacteria (CFU/ml), B) lactobacilli (CFU/ml), C) streptococci and staphylococci combined (CFU/ml), and D) *G. vaginalis* (CFU/ml) isolated from vaginal secretion with respect to Nugent scoring (median values indicated) (Kruskal-Wallis with Dunn's multiple comparison test).

TABLE 4. *Correlation between* G. vaginalis *and* L. crispatus, L. iners, L. gasseri, *or total lactobacilli*

G. vaginalis vs	Spearman correlation, *r*	*P* value
L. iners	−0.4733	0.0047
L. gasseri	ns	
L. crispatus	−0.3489	0.0431
Total lactobacilli	−0.3544	0.0397

gasseri was the most abundant *Lactobacillus* species in the intermediate group (6 out of 8 women) (Fig 2B). Furthermore, a negative correlation was found between *L. iners* and total bacterial count ($r=-0.5615$, $P=0.0006$) (not shown).

Correlation between concentrations of bacteria, with IL-1αlβ-, IL-6-, IL-8-, SLPI-, or endotoxin levels. In the cervical secretions, both IL-1α and IL-1β were significantly correlated with the total number of bacteria (Table 5).

L. iners showed an inverted correlation with cervical IL-1α, while *L. gasseri* and staphylococci were positively correlated with cervical IL-1β. In addition, both cervical and vaginal IL-8 correlated positively with staphylococci. All aerobic bacteria together (*Escheria coli*, *Streptococcus* sp., *Staphylococcus* sp., *Enterococcus faecalis*, *Corynebacterium* sp., *Brevibacterium*-like, *Aerococcus christensenii*) also correlated with cervical IL-8. IL-6 was detected only in a few women, thus giving no correlation.

As opposed to cervical IL-1α, cervical SLPI correlated positively with *L. iners* (Table 5).

Endotoxin in cervical secretion was associated with *L. gasseri*, aerobic bacteria, and GBS.

CDH

Semiquantitative analysis of 13 bacterial species. An example of a membrane with 12 vaginal and cervical samples is shown in Fig. 3. The membrane shows that patients no. 2, 5, 9, and 10 all had high numbers of *G. vaginalis* (G.v). The cervical concentrations of *G. vaginalis* were higher compared to those of vaginal samples in all except no. 10. High concentrations of *A. vaginae* were also seen in patients no. 9 and 10 with regard to both vaginal and cervical secretions. *P. bivia* was present only in patient no. 9. Only patient no. 9 was graded as BV.

Overall, the frequencies of women with any of the 13 bacterial species were higher by CDH than culture, except for *E. coli* (Fig. 4).

Concentration of bacteria in relation to Nugent scoring. Six of the eight typical BV-associated bacteria were correlated with Nugent score (Table 6). Four of the six bacterial species where cervical samples were compared still correlated with Nugent score.

Bacterial patterns of cervical and vaginal G. vaginalis, P. bivia, F. nucleatum *and* L. iners *with regard to the other 12 bacterial species.* With re-

TABLE 5. *Correlation between quantitative culture of vaginal samples and IL-1αlβ-, IL-8-, SLPI-, and endotoxin levels in either vaginal or cervical samples*

Bacterial spp.	Sampling site	Cytokine, endotoxin or SLPI	Spearman correlation, *r*	*P* value
CFU/g	Cervix	IL-1α	0.4165	0.0343
	Cervix	IL-1β	0.4890	0.0112
L. iners	Cervix	IL-1α	−0.4733	0.0047
	Cervix	SLPI	0.5735	0.0066
L. gasseri	Cervix	IL-1β	0.3997	0.0431
	Cervix	endotoxin	0.6362	0.0005
Staphylococci spp.	Cervix	IL-1β	0.4345	0.0266
	Cervix	IL-8	0.4118	0.0366
	Vagina	IL-8	0.4584	0.0095
S. agalactiae	Cervix	endotoxin	0.4073	0.0389
Aerobic bacterial spp	Cervix	IL-8	0.4942	0.0103
	Cervix	endotoxin	0.4585	0.0185

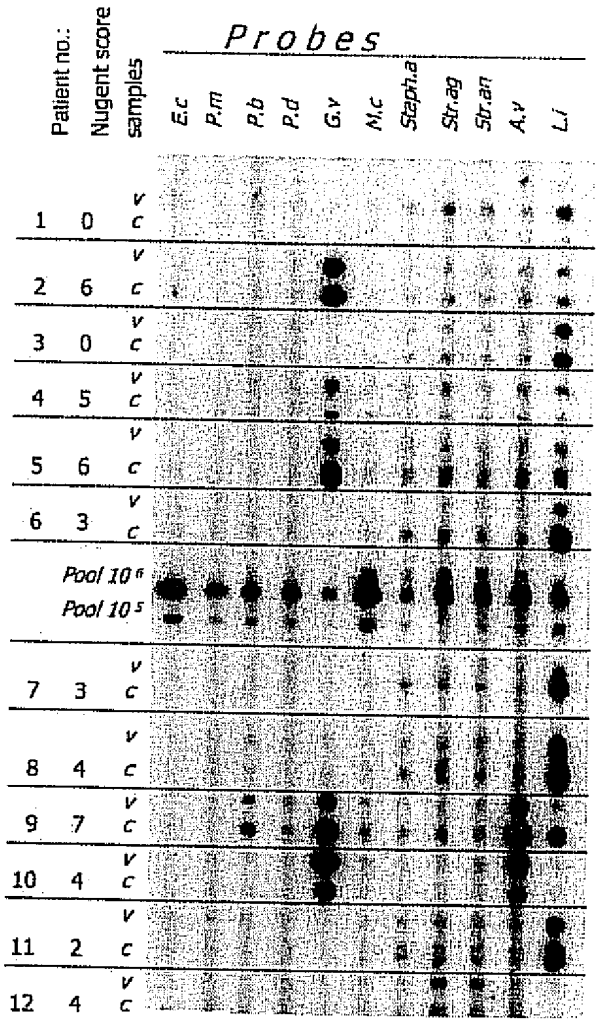

Fig. 3. CDH membrane showing the chemiluminescence intensity of 12 vaginal (v) and cervical (c) samples of women graded according to Nugent's scoring system using 12 probes (*E.c*=*E. coli*, *P.m*=*P. melaninogenica*, *P.b*=*P. bivia*, *P.d*=*P. disiens*, *G.v*=*G. vaginalis*, *M.c*=*M. curtisii*, *Staph. a*=*S. aureus*, *Str. ag*=*S. agalactiae*, *Str. an*=*S. anginosus*, *A.v.*=*A. vaginae*, *L.i*=*L. iners*). A standard pool of each bacterial species in an amount of 10^6 and $10^5$is also included (pool 10^6, pool 10^5). Semiquantitative estimation as described in Material and Methods.

gard to cervical bacteria, *G. vaginalis* correlated with *A. vaginae* and *P. bivia* with *P. disiens* and *B. ureolyticus* (Table 7). *F. nucleatum* was associated with *S. aureus* and *S. anginosus*. The only *Lactobacillus* species analyzed, *L. iners*, correlated with *S. aureus*.

Vaginal *G. vaginalis*, *P. bivia* and *F. nucleatum* were all significantly correlated with the five bacterial species: *A. vaginae*, *M. curtisii*, *B. ureolyticus*, *P. disiens* and *P. melaninogenica* (Table

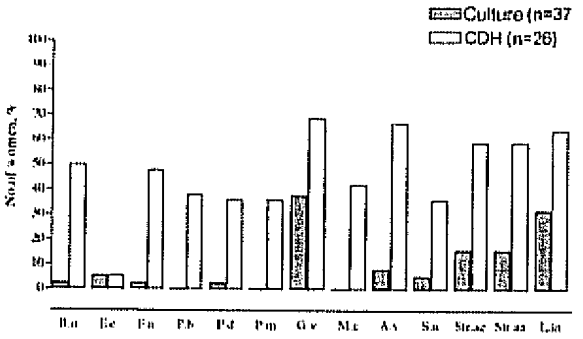

Fig. 4. Distribution (%) of 13 bacterial species in vaginal secretion analyzed by CDH or culture (see legend to Fig. 3 for abbreviations).

8). In addition, *G. vaginalis* was significantly associated with *P. bivia*. Furthermore, *F. nucleatum* was also correlated with *S. aureus*, *S. anginosus*, *S. agalactiae* and *L. iners*.

Correlations of bacterial species with IL-1αlβ, IL-6, IL-8, SLPI, or endotoxin. Analysis of correlations between the 13 bacterial species and the cytokines showed that in both cervix and vagina *B. ureolyticus* correlated with IL-1α, while a correlation was observed only in cervix with regard to IL-8 (Table 9). In addition, vaginal *F. nucleatum*, *S. agalactiae*, *S. anginosus* and *S. aureus* showed correlations with cervical IL-1α. The only correlation with IL-1β was seen with cervical *G. vaginalis*. An inverse correlation was found between *M. curtisii* and IL-1α in cervical fluid.

With regard to total Gram-negative bacteria in vaginal samples, SLPI showed a negative correlation and endotoxin a positive correlation (Table 9).

DISCUSSION

BV is a condition where the normal *Lactobacillus*-dominated biota is displaced by anaerobic bacteria. Although some of the most predominant bacterial species in asymptomatic women with BV are *G. vaginalis* and *Prevotella* sp., we have shown that other less known species such as *A. urogenitalis*, *A. vaginae*, and *P. harei* occur in concentrations exceeding 10^{11} CFU/ml vaginal secretion. Our results confirm the strong association of *A. vaginae* with BV (45, 46). *A. vaginae* has been implicated as a cause of salpin-

TABLE 6. *Correlation between bacterial species determined by CDH and Nugent score (Spearman rank correlation)*

Bacterial species *vs* Nugent score	No. of women analysed n	Vaginal fluid		Cervical fluid	
		r	*P* value	*r*	*P* value
P. bivia	26	0.5693	0.0024	0.5055	0.0230
P. disiens	22	0.6926	0.0004	0.5097	0.0437
P. melaninogenica	22	0.6448	0.0012	ns	
G. vaginalis	26	0.5855	0.0017	0.6387	0.0024
M. curtisii	26	0.6882	0.0001	ns	
A. vaginae	22	0.6380	0.0014	0.6439	0.0071

TABLE 7. *Correlation between bacteria in cervical secretions as measured by CDH*

Bacterial species	Correlation with	Spearman correlation, *r*	*P* value
G. vaginalis	*A. vaginae*	0.6451	0.007
P. bivia	*P. disiens*	0.9889	<0.0001
	B. ureolyticus	0.5408	0.0138
F. nucleatum	*S. anginosus*	0.7009	0.0025
	S. aureus	0.5710	0.0209
L. iners	*S. aureus*	0.7360	0.0012

TABLE 8. *Correlation of bacteria in vaginal secretions as measured by CDH*

Bacterial species	Correlation with	Correlation with Spearman correlation, *r*	*P* value
G. vaginalis	*A. vaginae*	0.6567	0.009
	M. curtisii	0.7061	0.0002
	B. ureolyticus	0.4461	0.0223
	P. disiens	0.5964	0.0034
	P. melaninogenica	0.7062	0.0002
	P. bivia	0.5279	0.0056
P. bivia	*A. vaginae*	0.4675	0.0283
	M. curtisii	0.6972	<0.0001
	B. ureolyticus	0.6123	0.0009
	P. disiens	0.6782	0.0005
	P. melaninogenica	0.5217	0.0128
F. nucleatum	*A. vaginae*	0.5061	0.0163
	M. curtisii	0.4968	0.0187
	B. ureolyticus	0.5610	0.0066
	P. disiens	0.7172	0.0002
	P. melaninogenica	0.4775	0.0246
	S. aureus	0.7397	<0.0001
	S. anginosus	0.4744	0.0257
	S. agalactiae	0.4476	0.0367
	L. iners	0.4647	0.0293
L. iners	*S. aureus*	0.7091	0.0002
	S. anginosus	0.6125	0.0024
	S. agalactiae	0.6167	0.0022

gitis and tubovarian abscesses (47, 48). In accordance with our results, our most recently characterized species *A. urogenitalis* was also observed in a BV patient by PCR (49, 50), while this is the first report on the concentration of this bacterium. The presence and amount of *P. harei* in BV has not been reported before. The very large numbers of these three fastidious bacterial species may have relevance for the treatment efficiency of BV. It is known for instance that *A. vaginae* is resistant to metronidazole (45, 51, 52). In general, the cure rate for BV at 4 weeks post treatment is estimated not to exceed 60–70% respecting both metronidazole and clindamycin (53). This low cure rate indicates that women with recurrent BV may benefit from a more detailed analysis of their cervicovaginal bacterial patterns.

Our findings confirm the correlation between Nugent score and total bacterial count, *G. vaginalis*, or total anaerobes (54). By CDH analysis, correlations were established between Nugent score and six out of the eight BV-associated bacteria included in the CDH. Correlations were also found between seven of these anaerobes. By CDH, fewer correlations were, however, found in cervical compared with vaginal samples. This may indicate that the host innate defense more strongly antagonizes the colonization of bacteria in the cervical canal. Overall, fewer correlations were found by culture than by CDH with regard to the eight BV-associated bacteria due to the low rate of positive cultures.

The correlations between the BV-associated bacteria measured by CDH were compatible with the correlations obtained with the Nugent score, except for *F. nucleatum* and *B. ureolyticus*. *F. nucleatum* correlated with *S. aureus*, *S. anginosus*, *S. agalactiae*, and *L. iners*, indicating an association with the intermediate and normal grades of Nugent score. It may help to explain the seemingly contradictory correlations of endotoxin with bacterial counts of *L. gasseri* and *S. agalactiae* (Table 5), since these bacteria were mostly presented within the intermediate grade and culture appeared to underestimate obligate anaerobic Gram-negative bacteria. Indeed, Gram-negative bacteria analyzed by CDH and taken together showed a correlation with the endotoxin in vaginal fluid.

Our results for *B. ureolyticus* and *F. nucleatum* are in agreement with the report by Hillier et al. (1). They observed an inverse relation of 10 BV-associated bacterial species to vaginal colonization of H_2O_2-producing lactobacilli except for these two bacterial species. *B. ureolyticus* has been suggested to be a commensal of the lower genital tract of both men and women (4). Furthermore, these two species are also common isolates in occult amniotic fluid infection with intact fetal membranes (21).

The most frequent bacterial species were *L. gasseri*, *G. vaginalis* and *L. iners*. The intermediate grade was characterized by the highest frequency and numbers of *L. gasseri* and streptococci/staphylococci. Among the women with this grade, *L. gasseri* as well as streptococci or *G. vaginalis* occurred as the dominating vaginal species of the total bacterial count. Several women with a Nugent score of 6 appeared to have high concentrations of *G. vaginalis*, some in combination with high numbers of *A. vaginae* (Fig. 3). The intermediate grade has been regarded as being a transition phase either leading to BV or returning to a normal grade (54, 55). Our findings suggest, however, that this grade includes various bacterial patterns, one of which may be a transition phase, while others may be entities of their own. Our culture results are better reflected by the scoring of Gram-stained vaginal smears according to Ison & Hay, since they included two more categories as compared to Nugent scoring, one of which was defined by high concentrations of streptococci or staphylococci (56). The occurrence of *L. gasseri*, *G. vaginalis* or streptococci as the predominating species in women graded as intermediate according to Nugent scoring indicates that the method is inadequate for defining entities which are not BV or typical *Lactobacillus*-dominated biota.

A more vaginitis-like condition has been reported that was correlated with a bacterial microbiota mainly consisting of aerobic bacteria such as group B streptococci, *S. aureus*, and *E. coli* (57). *G. vaginalis* was also observed in a fraction of these patients. The term aerobic vaginitis was used to describe this grade of abnormal vaginal microbiota in combination with symptoms. Our bacterial patterns in the intermediate grade showed some similarities with this aerobic vaginitis-type microbiota, although clinical symptoms were lacking.

Few studies have compared species-specific

Lactobacillus counts in normal and intermediate grades. *L. iners* and *L. crispatus*, in contrast to *L. gasseri*, were inversely correlated with *G. vaginalis*. Furthermore, *L. gasseri* as opposed to *L. iners* is a H_2O_2-producing *Lactobacillus* species. Thus, with regard to the lacking H_2O_2 production in *L. iners*, there seem to be other factors that equally well promote a *Lactobacillus*-dominated vaginal microbiota. Another interpretation is that H_2O_2 is less important for sustaining a *Lactobacillus*-dominated microbiota (58). The finding of *L. gasseri*, *L. iners*, *L. crispatus*, and *L. jensenii* as the predominating lactobacilli among asymptomatic women is in agreement with the findings of others (59, 60).

A foul smell with clinical symptoms of inflammation is the most prominent sign of symptomatic BV. Despite this absence of inflammation, BV is characterized by increased cytokine levels of IL-1 and IL-8, but not IL-6 (23, 24, 61). In fact, our finding of a correlation between total CFU and cervical IL-1α/β could be interpreted as though the IL-1 levels are a consequence of the vaginal bacterial load in the non-inflammatory condition, escalating the alert status of the host (Table 5). The correlation between *G. vaginalis*, as analyzed by CDH, and IL-1, is thus an expected finding. As reported by Donders et al., women with vaginitis carrying mainly bacteria such as *S. aureus*, group B streptococci, and *E. coli* expressed higher vaginal IL-1 levels than either normal women or women with BV (57). In our study, both culture and CDH indicated that staphylococci and streptococci may stimulate increased cytokine secretion (IL-1 and IL-8) in asymptomatic healthy women (Tables 5 & 9). *B. ureolyticus* and *F. nucleatum*, as analyzed by CDH, correlated with vaginal IL-1. *B. ureolyticus* was also associated with cervical IL-1α and IL-8. The vaginal presence of these two strains has been reported to be associated with preterm delivery, amniotic fluid infection or elevated IL-6 levels in the amniotic fluid (17, 62). It appears that microbial patterns or single bacterial species, including several strict anaerobic Gram-negative bacteria, need to be related to the grade of clinical and laboratory inflammatory signs in order to clarify whether there are distinct entities of abnormal vaginal microbiota leading to somewhat different host responses, especially respecting intrauterine subclinical infection in pregnant women.

A factor that could influence growth of bacteria in the genital tract is SLPI, since it expresses antimicrobial activity (31, 33). Of the four most common *Lactobacillus* species, a correlation was only established between *L. iners* and cervical SLPI. By CDH analysis, an inverted correlation was obtained between vaginal Gram-negative bacteria and SLPI. These results suggest that the secretion of SLPI is affected by both the number and type of bacteria. It is plausible that as SLPI expresses antibacterial activity it becomes bound to bacteria and thereby "consumed" by high bacterial concentrations, consequently decreasing the levels in vaginal secretion. Alternative explanations could be microbial degradation of SLPI as well as downregulation by the host in response to a disturbed vaginal microbiota. Our results are in agreement with the findings of Draper et al. (27), showing a reduction of vaginal SLPI in women with BV. The mechanism for this relationship between bacteria and host innate re-

TABLE 9. *Correlation between vaginal / cervical bacteria, as measured by CDH, and the levels of cytokines, endotoxin, and SLPI*

Bacterial species	Sampling site	Cytokine, endotoxin or SLPI	Spearman correlation, *r*	*P* value
B. ureolyticus	cervical	IL-1α	0.4985	0.0253
	cervical	IL-8	0.4871	0.0294
	vaginal	IL-1α	0.4514	0.0268
F. nucleatum	vaginal	IL-1α	0.4994	0.0250
all Gram neg.	vaginal	SLPI	−0.5795	0.0299
	cervical	endotoxin	0.4467	0.0483
M. curtisii	cervical	IL-1α	−0.5507	0.0119
G. vaginalis	cervical	IL-1	0.4615	0.0405
S. agalactiae	vaginal	IL-1α	0.5536	0.0113
S. anginosus	vaginal	IL-1α	0.5846	0.0068
S. aureus	vaginal	IL-1α	0.4667	0.0380

sponses is of interest, especially since both BV and SLPI have been discussed in the context of HIV infection. BV increases the susceptibility to HIV, while high levels of SLPI have been suggested to reduce the risk of contracting HIV (12, 14, 37, 63).

Concerning the CDH results, the correlations obtained between bacteria and cytokines, endotoxin, or SLPI did deviate somewhat from those obtained by culture. One explanation is that more samples containing anaerobic bacteria were identified with this method. In addition, somewhat fewer patients were analyzed by CDH and the CDH analyses were not titrated, meaning that samples exceeding 10^6 bacteria were given the same values.

By CDH, it seems possible to semiquantitatively analyze vaginal and cervical samples of patients with respect to many fastidious and strictly anaerobic bacteria. It thus appears that CDH is a suitable tool for future studies on various patient groups with the aim of quantifying 30–40 separate bacterial species without the laborious task of culture and identification. Such a clinical group may be women with recurrent BV, where knowledge of the vaginal bacteriologic status could be helpful when deciding on adequate antibiotic treatment. By analysis of cervicovaginal specimens with CDH, the inherited bias of culture due to suboptimal media for various bacterial species can be avoided. Likewise, the effect on viability of sometimes long periods in transport medium before the samples reach the laboratory would be of less importance for the analysis.

BV is associated with upper genital tract infections, spontaneous preterm delivery, spontaneous abortion, and increased susceptibility to HIV and other STDs. More information about the quantitative composition and patterns of microorganisms of the vagina and their relationship to the local host inflammatory response could help our understanding of the mechanism(s) leading to disturbed vaginal microbiota, subclinical ascending infections, and reduced resistance to STDs. By defining a risk group of women on the basis of bacteriologic diagnosis and host immune factors, antibiotic treatment could be made more specific, possibly increasing the cure rate in women with a disturbed vaginal microbiota.

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EXHIBIT F

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Anti-inflammatory effects of probiotic yogurt in inflammatory bowel disease patients

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Summary

Our aim was to assess anti-inflammatory effects on the peripheral blood of subjects with inflammatory bowel disease (IBD) who consumed probiotic yogurt for 1 month. We studied 20 healthy controls and 20 subjects with IBD, 15 of whom had Crohn's disease and five with ulcerative colitis. All the subjects consumed *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 supplemented yogurt for 30 days. The presence of putative regulatory T (T_{reg}) cells ($CD4^+$ $CD25^{high}$) and cytokines in T cells, monocytes and dendritic cells (DC) was determined by flow cytometry from peripheral blood before and after treatment, with or without *ex vivo* stimulation. Serum and faecal cytokine concentrations were determined by enzyme-linked immunosorbent assays. The proportion of $CD4^+$ $CD25^{high}$ T cells increased significantly ($P = 0{\cdot}007$) in IBD patients, mean (95% confidence interval: CI) 0·84% (95% CI 0·55–1·12) before and 1·25% (95% CI 0·97–1·54) after treatment, but non-significantly in controls. The basal proportion of tumour necrosis factor (TNF)-α^+/interleukin (IL)-12^+ monocytes and myeloid DC decreased in both subject groups, but of stimulated cells only in IBD patients. Also serum IL-12 concentrations and proportions of IL-2^+ and $CD69^+$ T cells from stimulated cells decreased in IBD patients. The increase in $CD4^+$ $CD25^{high}$ T cells correlated with the decrease in the percentage of TNF-α- or IL-12-producing monocytes and DC. The effect of the probiotic yogurt was confirmed by a follow-up study in which subjects consumed the yogurt without the probiotic organisms. Probiotic yogurt intake was associated with significant anti-inflammatory effects that paralleled the expansion of peripheral pool of putative T_{reg} cells in IBD patients and with few effects in controls.

Keywords: anti-inflammatory, inflammatory bowel disease, probiotics

Introduction

There is evidence to suggest that probiotic bacteria may have, in a species- or even strain-dependent manner, a potential use as anti-inflammatory agents in some chronic inflammatory diseases [1,2]. The most promising clinical results have been obtained in the prevention and management of atopic eczema and the management of inflammatory bowel disease (IBD) and post-operative pouchitis [3,4]. On the basis of experimental data, the anti-inflammatory effects of probiotics may be a consequence of antagonism against potentially pathogenic/proinflammatory endogenous microbiota; modulation of the balance between T helper 1 (Th1), Th2 and regulatory T (T_{reg}) cells; down-regulation of proinflammatory [e.g. interleukin (IL)-12, tumour necrosis factor (TNF)-α] and/or stimulation of anti-inflammatory (e.g. IL-10) cytokine production; as well as effects seen such as enhanced elimination, modified degradation, permeation and presentation of proinflammatory antigens [3–5].

Data accumulated in the past few years have emphasized the central role of T_{reg} cells in the formation and maintenance of tolerance to mucosally encountered antigens and down-regulation of ongoing inflammation [6,7]. Accordingly, their deficient activity, in contrast to enhanced Th1 or Th2 action, has been implicated in chronic inflammatory conditions, including allergic diseases and IBD [7–9]. Dendritic cells (DC) are thought to be the primary regulators in determining the balance between the different T cell subtype activities in a manner that is dependent upon multiple factors, including the DC subtype and local cytokine milieu,

with the presence of IL-10 and IL-12 having particular importance [10–12]. Interestingly, a recent study in mice demonstrated that probiotic bacteria may confer protection against chemically induced intestinal inflammation by induction of T_{reg} cells [5]. Whether any probiotic strains have this ability in humans is unknown, but is feasible based upon *in vitro* data and indirect evidence from clinical studies demonstrating that the intake of certain probiotics may enhance the production of IL-10 [13] and transforming growth factor (TGF)-β [14,15] which could, theoretically, promote the induction of T_{reg} cells or be indicative of enhanced T_{reg} function [16–18].

The aim of the current study was to assess whether intake of yogurt supplemented with two probiotic strains, *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14, with documented efficacy in controlling mucosal infections [19] and the ability to pass through the gastrointestinal tract alive, may promote an anti-inflammatory immunological milieu in subjects with active chronic inflammatory conditions, namely Crohn's disease or ulcerative colitis. These conditions are characterized by chronic intestinal inflammation that is thought to result from exaggerated effector T cell responses towards endogenous bacterial antigens [9]. There were two reasons for using a yogurt delivery system. First, patients with chronic inflammation are often receiving a range of pharmaceutical agents, some of which have side effects including diarrhoea and loss of appetite. Potentially, yogurt could provide an excellent nutritional supplement that reduced diarrhoeal problems. Secondly, we wanted to test the effect of probiotic yogurt on peripheral immunity as a step towards taking this food to populations that have little access to, and no buying power for the purchase of, pharmaceutical products. One such application is to developing countries, and to that end we have established active collaborations in Tanzania and Nigeria where, in the former case, local people have begun to produce the probiotic yogurt themselves in order to support the health of their families and community (http://www.westernheadseast.ca).

Materials and methods

Subjects

It should be noted that the principal aim of this project was not to cure IBD or study clinical outcomes, as the aim was to determine if any effects arose with a nutritional change that did not require any alteration in the standard medical management of the patient. To that end, no effort was made to control for use of steroids, although no subjects received antibiotics during the study. The study population comprised 20 subjects with IBD and 20 healthy controls with no known or suspected intestinal abnormalities. The mean age ± standard deviation (s.d.) of IBD patients was 44 ± 11·7 (range 26–63) years and that of controls 51 ± 6·4 (38–61) years. Of the IBD patients, 15 had Crohn's disease, five had ulcerative colitis and all had subjective symptoms, including liquid or very soft stools and/or abdominal pain, indicative of active IBD. To reduce patient-to-patient variability, all the subjects were women. Exclusion criteria included pregnancy, use of antibiotics, lactose intolerance and premature termination of the study (only three of 23 healthy subjects were excluded due to inability to comply with the study protocol). All subjects were asked to continue with their habitual diet but to refrain from taking any other yogurt or probiotic supplements 2 weeks before and during the study. The patient group did not alter any ongoing medication being given for their IBD. Informed consent was obtained from all subjects and the study was approved by the Review Board for Health Sciences Research involving Human Subjects, at the University of Western Ontario, London, Ontario, Canada.

Design

In this open-label study, all subjects consumed 125 g of probiotic yogurt per day for 30 days. The researchers were blinded regarding the study groups. To rule out the influence of yogurt alone, the treatment regimen was repeated in an exploratory study using unsupplemented yogurt with a subpopulation of the same IBD patients ($n = 8$; six with Crohn's disease, two with ulcerative colitis) after a washout period of 6 months.

The main outcome parameters measured were changes in the prevalence of putative T_{reg} cells ($CD4^+$ $CD25^{high}$) and TNF-α- and IL-12-producing monocytes and DC in peripheral blood (PB) during treatment. Secondary outcome measures included changes in the presence of T cell surface activation markers, serum and faecal cytokine concentrations and *ex vivo* proliferative responses of PB mononuclear cells (PBMC). Individual stool and blood samples were collected before (day 0) and after (day 30) the treatment period.

The patients were asked to note in a diary any changes in symptoms, including bloating, gas, abdominal pain and constipation/loose stools throughout the study as possible side effects of the yogurt consumption.

Preparation of probiotic yogurt

To prepare a probiotic mother culture, dried *L. rhamnosus* GR-1 (GR-1) and *L. reuteri* RC-14 (RC-14) were added to Man, Rogosa and Sharpe broth (EM Science, Gibbstown, NJ, USA) at a rate of 1·5% and grown anaerobically at 37°C overnight. Then a mixture of milk (1% fat), 0·33% yeast extract and 0·4% inulin was autoclaved for 15 min, cooled to 37°C, and inoculated with the probiotic culture at a rate of 1% and incubated anaerobically at 37°C overnight.

To prepare probiotic yogurt, a mixture with milk (1% fat) and 5% sugar was heat-treated at 87°C for 30 min, cooled to 37°C, inoculated with 4% of the probiotic mother culture

and 2% of standard plain yogurt containing *L. delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, fermented at 37°C for 6 h and stored at 4°C. After 2 days 11% strawberry flavouring (Sensient, Rexdale, ON, Canada) was added and the yogurts were packaged. Viable counts and quality assurance was tested at regular intervals. A new batch of yogurt was produced every 2 weeks to ensure consistency in viable counts of probiotic bacteria, especially as those of RC-14 decreased rapidly with time. After 2 weeks at 4°C the total counts were consistently at 1×10^7 for RC-14 and 2×10^7 colony-forming units (cfu)/ml for GR-1. No contaminants were isolated at any time in the study.

Analysis of intracellular cytokine production

Intracellular cytokine detection was performed by flow cytometry as described previously, with some modifications [20,21]. PB samples in lithium heparin were supplemented one-to-one with RPMI-1640 medium (Invitrogen, Burlington, ON, Canada), incubated at 37°C in a 5% CO_2 humidified atmosphere with brefeldin A (10 μg/ml, Sigma, St Louis, MO, USA) in the presence or absence of lipopolysaccharide (LPS, 100 ng/ml; from *Escherichia coli*, serotype 055:B5, Sigma) plus interferon (IFN)-γ (100 units/ml; R&D Systems, Inc., Minneapolis, MN, USA) for stimulation (6 h) of cytokine production by monocytes and DC; ionomycin (1 μg/ml, Sigma) plus phorbol 12-myristate 13-acetate (PMA, 25 ng/ml, Sigma) for stimulation (4 h) of cytokine production by T cells. For identification of the whole DC population [major histocompatibility complex (MHC) II$^+$/lineage-/CD33$^{+/-}$], their highly and intermediately CD33-expressing myeloid (CD33^{high}, CD33^{intermed}) and no or weakly CD33-expressing plasmocytoid (CD33$^{-/low}$) subsets and monocytes (MHC II$^+$/CD14$^+$/CD33$^+$), PB cells were then incubated for 15 min at room temperature (RT) with anti-human leucocyte antigen D-related (HLA-DR)-Cy-chrome, anti-CD33-allophycocyanin (APC) and each of the following fluorescein isothiocyanate (FITC)-labelled lineage marker antibodies: anti-CD3, anti-CD19, anti-CD56 and anti-CD14 (BD Biosciences, San Diego, CA, USA). Stained cells were washed with phosphate-buffered saline (PBS, pH 7·5) and centrifugation (5 min at 540 *g*), fixed, permeabilized and stained with anti-TNF-α-phycoerythrin (PE, clone MAb11) and anti-IL-12-PE (C11·5) using the Fix & Perm reagent (Caltag, Burlingame, CA, USA) following the manufacturer's instructions. T cell cytokines were analysed accordingly, but the cells were identified with anti-CD3-FITC and their cytokines detected with anti-IL-2-PE (clone MQ1-17H12), anti-IFN-γ-PE (B27), anti-IL-4-PE (8D4-8) and anti-IL-10-PE (JES3-19F1). Data acquisition was performed in two consecutive steps with a flow cytometer (FACSCalibur™, BD Biosciences). First, 30 000 events/test corresponding to the whole PB cellularity were collected for analysis of cytokines produced by T cells and monocytes. Secondly, only events in a HLA-DR$^+$/CD3$^-$/CD19$^-$/CD56$^-$/CD14$^-$ live gate were stored and a minimum of 300 000 events from the total PB cellularity were acquired in order to obtain at least 1000 MHC II$^+$/lineage$^-$ cells for the analysis of cytokines produced by DC subsets. CellQuest™ software (BD Biosciences) was used for data acquisition and analysis. Representative acquisition dot plots demonstrating the identification of monocytes and DC are presented in Fig. 1.

Analysis of T cell surface markers

For the expression of early activation marker CD69 on T cells, RPMI-1640 diluted PB was incubated with or without PMA and ionomycin as described above, whereas only the unstimulated sample was used for T_{reg} cell analysis. The percentage of CD4$^+$ CD25$^+$ T_{reg} cells are enriched within the 1–2% of PB CD4$^+$ T cells expressing high levels of CD25, while the population expressing lower levels of CD25 is thought to consist mainly of activated effector T cells [22]. Thus, using flow cytometry we gated on small lymphocytes and CD4$^+$ T cells were subdivided into bright (CD4$^+$ CD25^{high}/T_{reg}) and intermediate (CD4$^+$ CD25$^+$/activated T cell) populations based on their CD25 expression (Fig. 2a). The stimulated and/or unstimulated samples (200 μl each) were stained with 3 μl of anti-CD3-FITC in combination with anti-CD69-PE or anti-CD4-FITC plus anti-CD25-PE (BD Biosciences) for 15 min at RT. Data were acquired with a flow cytometer (30 000 events/test) and analysed as described above.

Enzyme-linked immunosorbent assays (ELISA)

Faecal extracts were prepared by mixing 3 g of stool with 3 ml of PBS followed by centrifugation (30–45 min at 20 000 *g*) at 4°C and filtration of the supernatant through a 0·45-μm pore-size filter [23]. Serum samples and faecal extract aliquots were stored at −70°C until analysis. The concentrations of TNF-α, IL-12 and IL-10 were measured with BD OptEIA™ ELISA sets (BD Biosciences) according to the manufacturer's instructions.

Proliferation assay

Cell-free extracts (CFE) of RC-14 and GR-1 were prepared from capsules containing 1×10^9 cfu of RC-14 and GR-1 [24,25]. The bacteria were washed twice and suspended in PBS (1 ml) and then bead-beaten with 300 mg of zirconium beads (0·1 mm) (3 min at 2300 *g*) using a mini-bead beater (Biospec Products, Bartlesville, OK, USA). Particulates were removed by centrifugation (10 min at 12 000 *g*) and the protein concentration in the supernatants (CFE) determined with the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA), with bovine serum albumin as the protein standard. PBMC were isolated from PB in sodium heparin by Ficoll-Hypaque (Pharmacia Biotech,

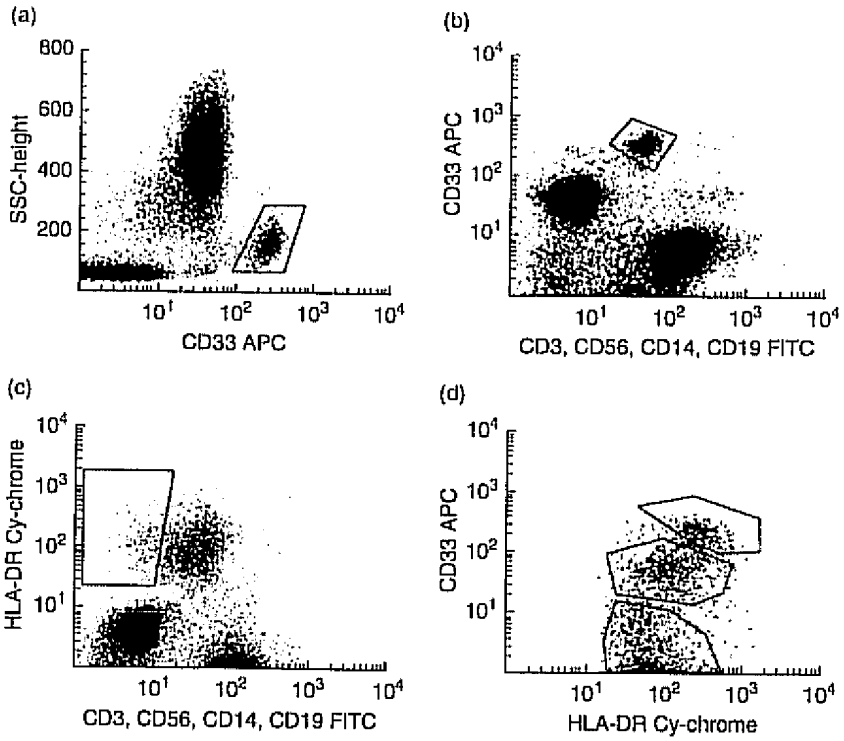

Fig. 1. Identification of monocytes and dendritic cells (DC) by flow cytometry. A representative example of the identification of monocytes based on the expression of (a) CD33 and (b) CD14 antigen. (c) Identification of DC as a human leucocyte antigen D-related (HLA-DR)$^+$ lineage$^-$ (CD3$^-$, CD56$^-$, CD14$^-$, CD19$^-$) population. (d) After acquiring a higher number of cells within the HLA-DR$^+$ lineage$^-$ live gate, three different dendritic cell subsets were identified on the basis of CD33 expression: myeloid CD33^{high}, CD33^{intermed} and plasmocytoid CD33$^{-/low}$.

Uppsala, Sweden) gradient centrifugation. PBMC ($0{\cdot}5 \times 10^6$/ml) were cultured in RPMI-1640 with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% fetal bovine serum supplemented with CFE in the presence or absence of ionomycin (100 ng/ml) plus PMA (100 ng/ml) for 4 days at 37°C in a 5% CO_2 humidified atmosphere. Cultured cells were then incubated further on 96-well plates (200 µl/well in triplicate) for 4 h at 37°C with 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) (2·5 mg/ml in PBS) per well. The plates were centrifuged (5 min at 500 *g*) and supernatants were removed. Hydrochloric acid (HCL) (0·04 N) in isopropanol (100 µl) was added to each well and absorbance measured at 575 nm (reference wavelength 650 nm) with a microplate reader (Bio-Rad Model 550).

Statistics

Statistical analysis was performed with GraphPad Prism® version 4 (GraphPad, Software, Inc., San Diego, CA, USA) and StatView® version 4·57 (Abacus Concepts, Inc., Berkeley, CA, USA) with the exception of the Exact unconditional test for 2×2 tables, which was used for comparing frequency of symptoms before and after treatment [26]. Changes in immunological measurements between two time-points within a subject group were compared with the paired two-tailed *t*-test if the data were parametric with or without natural logarithmic transformation and by the Wilcoxon signed-rank test if the data were nonparametric and non-transformable. Differences between subject groups were compared with the unpaired two-tailed *t*-test if the data were parametric and with Mann–Whitney *U*-test if the data were nonparametric and non-transformable. Correlations between two continuous variables were analysed by Spearman's rank correlation test. *P*-values < 0·05 were considered statistically significant.

Results

Effect of probiotic yogurt intake on T cells

The percentage of CD4$^+$ CD25^{high} cells increased significantly following treatment with probiotic yogurt from the group mean (95% confidence interval, CI) of 0·84% (95% CI 0·55–1·12) to 1·25% (95% CI 0·97–1·54) ($P = 0{\cdot}007$, Fig. 2b). In controls the response was significantly different ($P = 0{\cdot}03$) with little increase from before, 0·69% (95% CI 0·50–0·87%95% CI), to after, 0·73% (95% CI 0·59–0·87) the treatment ($P = 0{\cdot}09$) (Fig. 2c). Similarly, the change in the percentage of CD4$^+$ CD25$^+$ T cells was significantly different between the groups ($P = 0{\cdot}01$) with an increase from 9·1% (95% CI 7·2–11·0%95% CI) to 11·0% (95% CI 9·5–13·1) ($P = 0{\cdot}003$, Fig. 2c) in IBD patients and no change from before, 6·68% (95% CI 5·78–7·59), to after, 6·47% (95% CI 5·69–7·24) the treatment in controls ($P = 0{\cdot}36$, Fig. 2e).

In IBD patients, but not in controls, the treatment was followed by a reduced percentage of CD3$^+$ T cells responding to polyclonal *ex vivo* stimulation by production of IL-2. In IBD patients the mean percentage of IL-2$^+$ CD3$^+$ T cells was 42·3% (95% CI 35·4–49·2) before and 38·2% (32·2–44·2%)

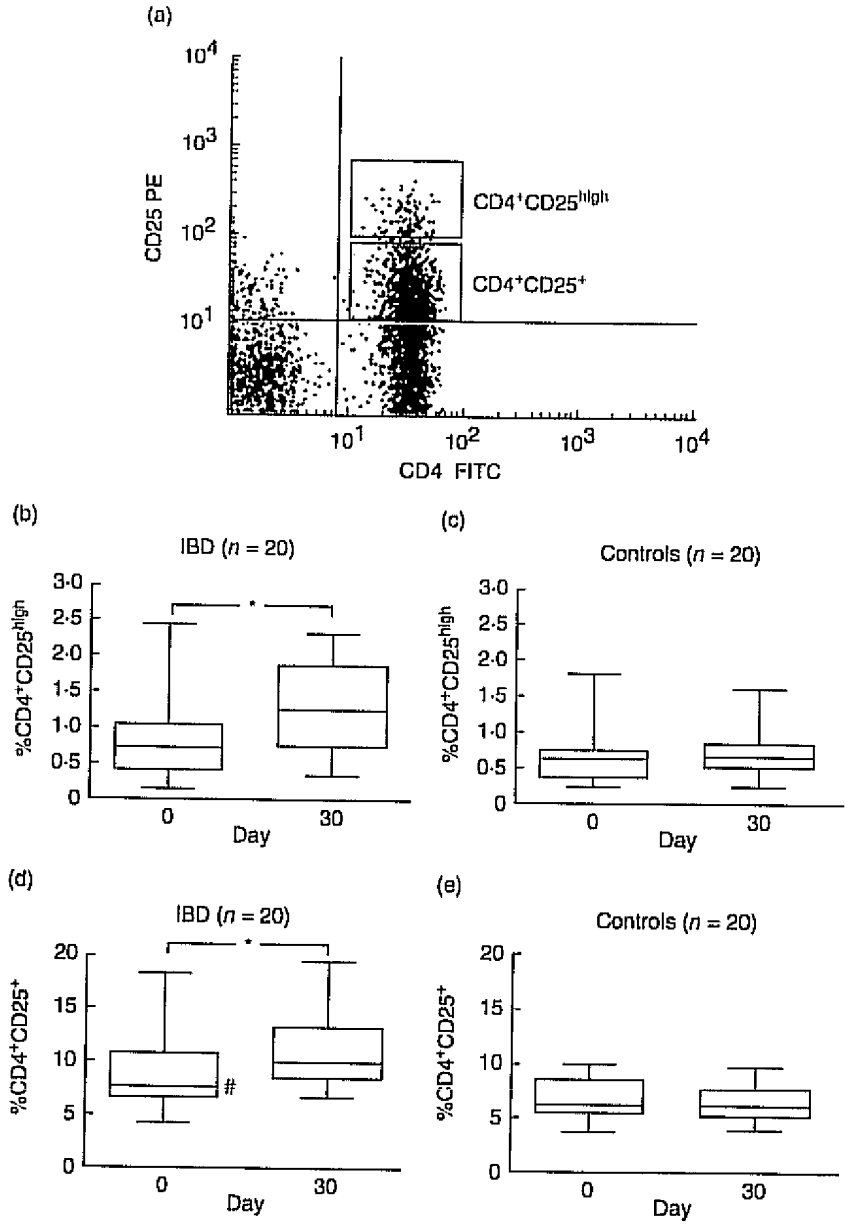

Fig. 2. (a) A representative example of the analysis of $CD4^+$ $CD25^+$ cells by flow cytometer. Lymphocytes were gated according to their particular small forward-/side-scatter profile and the $CD4^+$ $CD25^{high}$ and $CD4^+$ $CD25^+$ cells identified based on the concentration of CD25 expression. (b–e) Percentage of $CD4^+$ $CD25^{high}$ and $CD4^+$ $CD25^+$ cells in peripheral blood before (0 day) and after (30 days) probiotic yogurt treatment in inflammatory bowel disease (IBD) patients and controls. Data are shown as box plots with median and 10th, 25th, 75th and 90th percentiles. *Significant ($P < 0{\cdot}05$) increase in $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^{high}$ cells in IBD patients following treatment. #The basal (0 day) percentage of $CD4^+$ $CD25^+$ cells was significantly higher in IBD patients than in controls ($P = 0{\cdot}04$).

($P = 0{\cdot}03$) after the treatment, while the respective values for controls were 42·4% (95% CI 36·7–48·0) and 44·4% (95% CI 39·3–49·4) ($P = 0{\cdot}50$). The difference in the change between the groups was not significant ($P = 0{\cdot}20$). No other significant effects were observed in the intracellular cytokine production by $CD3^+$ T cells (data not shown). However, the percentage of stimulated T cells expressing CD69 decreased in the IBD patients ($P = 0{\cdot}02$), but again, not in the control group ($P = 0{\cdot}77$, Fig. 3a,b). This difference between the groups approached statistical significance ($P = 0{\cdot}07$).

Effect of probiotic yogurt intake on monocytes and DC

The basal proportion (before treatment) of monocytes and DC which produced TNF-α or IL-12 was higher in the IBD patients compared to controls, with some differences reaching statistical significance ($P < 0{\cdot}05$; Table 1). The proportion of monocytes or DC populations in PB *per se* did not change following treatment with probiotic yogurt, whereas significant decreases were observed in the percentages of unstimulated TNF-α- and IL-12-producing monocytes and myeloid DC subsets in both IBD patients and controls, as summarized in Table 1. In unstimulated and/or stimulated plasmocytoid DC subset the production of these cytokines was very low or undetectable with no significant changes during the treatment (data not shown). Significant correlations were observed between the change in the proportion of T_{reg} cells (increase) following the treatment and the change (decrease) in the proportion of unstimulated TNF-α- and IL-12-producing monocytes ($\rho = -0{\cdot}59$, $P = 0{\cdot}01$ and

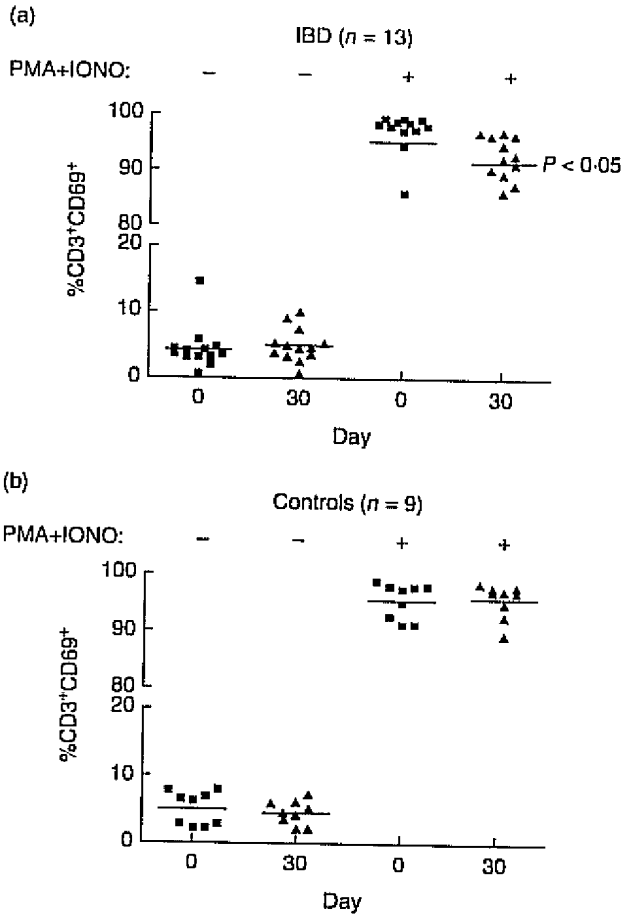

Fig. 3. The percentage of $CD3^+$ $CD69^+$ T cells in peripheral blood before (day 0) and after (day 30) probiotic yogurt treatment in inflammatory bowel disease (IBD) patients and controls with (+) or without (–) *ex vivo* stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin. The horizontal bars represent mean values.

$\rho = -0{\cdot}58$, $P = 0{\cdot}01$, respectively) and DC ($\rho = -0{\cdot}53$, $P = 0{\cdot}02$ and $\rho = -0{\cdot}61$, $P = 0{\cdot}008$, respectively) (Fig. 4).

Effect of probiotic yogurt intake on serum and stool cytokines

The serum IL-12 concentration decreased significantly in both IBD patients and controls following the intake of probiotic yogurt, the group mean (95% CI) decreasing from 51·6 (95% CI 38·4–64·8) to 44·9 (95% CI 34·5–55·4) pg/ml ($P = 0{\cdot}02$) in IBD patients and from 50·1 (95% CI 41·5–58·8) to 46·1 (95% CI 38·9–53·3) pg/ml in controls ($P = 0{\cdot}03$). The concentrations of TNF-α and IL-10 were variable in IBD patients and no significant changes were observed (data not shown). In controls, the serum concentrations of TNF-α decreased from group mean (95% CI) 7·6 (95% CI 4·7–10·5) to 5·6 (95% CI 3·4–7·8) pg/ml ($P = 0{\cdot}002$), while the faecal concentrations increased from 9·3 (95% CI 3·6–15·0) to 14·2 (95% CI 5·6–22·9) pg/ml ($P = 0{\cdot}006$) after treatment.

Patient diaries

Analysis of patient diaries revealed two findings. One of 20 IBD patients reported excess intestinal gas at the time of recruitment and six at the end of the treatment period ($P = 0{\cdot}02$), while one of 20 reported subjectively low abdominal pain at the recruitment and six at the end of the treatment period ($P = 0{\cdot}02$). These latter six patients had significantly lower mean (95% CI) faecal concentrations of IL-12, 9·1 (95% CI 0·65–17·5) than the remaining IBD patients ($n = 14$), 13·0 (95% CI 8·9–17·0) pg/ml ($P = 0{\cdot}04$) at the end of the treatment period. No other significant changes or correlations with immunological variables were noted regarding the subjective symptoms.

In vitro proliferative responses of PBMC to CFE of RC-14 and GR-1

Addition of RC-14/GR-1 CFE to PBMC cultures induced only a marginal increase in proliferation compared to unstimulated PBMC from healthy controls, whereas it appeared to inhibit the PMA + ionomycin-induced proliferation (Fig. 5). Similar results were seen with PBMC from IBD patients and controls before and after consumption of probiotic yogurt (data not shown).

Immunomodulatory properties of unsupplemented yogurt

In the follow-up of eight IBD patients no significant changes were observed in the percentage of T_{reg} cells, activated T cells or TNF-α/IL-12-producing monocytes or DC following the 30-day intake of unsupplemented yogurt. The lack of changes were contrary to the significant changes that followed the intake of probiotic yogurt, as indicated in Fig. 6.

Discussion

The results of this study demonstrate that the consumption of probiotic yogurt can result in an increased proportion of putative $CD4^+$ $CD25^+$ T_{reg} cells ($CD4^+$ $CD25^{high}$) in the peripheral blood of IBD patients. This effect has not been reported previously in humans, although a recent study in a mouse colitis model showed that a dried probiotic cocktail protected against chemically induced intestinal inflammation by the induction of $CD4^+$ TGF-β-bearing T_{reg} cells [5]. Expansion of the peripheral pool of $CD4^+$ $CD25^{high}$ cells is particularly interesting in light of recent data, suggesting that in active Crohn's disease and ulcerative colitis these cells have adequate suppressive function, but the peripheral pool is numerically insufficient in supplying enough of them to the intestinal inflammatory lesions [27]. The expansion of peripheral $CD4^+$ $CD25^+$ T_{reg} cells in IBD patients may therefore have fundamental importance for promoting and maintaining remission. It is noteworthy that the percentages of

Table 1. The *ex vivo* intracellular production of tumour necrosis factor (TNF)-α and interleukin (IL)-12 by unstimulated and stimulated peripheral blood (PB) monocytes and dendritic cells (DC) from inflammatory bowel disease (IBD) patients and controls before and after treatment with probiotic yogurt.

	% Cells in total in PB (mean ± s.e.)/% cytokine-producing cells (mean ± s.e.)			
	IBD patients (n=20)		Controls (n=20)	
Cell type/cytokine	Before treatment	After treatment	Before treatment	After treatment
Monocytes	4·9 ± 0·4	4·4 ± 0·4	4·0 ± 0·3	3·9 ± 0·4
TNF^+ basal[a]	6·4 ± 2·4[c]	1·6 ± 0·5[d]	2·7 ± 0·4	1·5 ± 0·3[e]
TNF^+ stimulated[b]	58·1 ± 4·7	49·7 ± 3·3[d]	50·7 ± 4·2	49·6 ± 3·7
$IL\text{-}12^+$ basal	3·4 ± 0·7	1·5 ± 0·3[d]	2·1 ± 0·2	1·2 ± 0·2[d]
$IL\text{-}12^+$ stimulated	21·7 ± 2·5	16·3 ± 2·0[d]	17·2 ± 2·5	14·2 ± 2·5
Dendritic cells (all)	0·7 ± 0·1	0·7 ± 0·1	0·8 ± 0·1	0·7 ± 0·1
TNF^+ basal	5·9 ± 1·7[c]	1·4 ± 0·3[d]	2·2 ± 0·3	1·2 ± 0·2[e]
TNF^+ stimulated	35·9 ± 3·5	27·3 ± 2·0[d]	26·8 ± 3·7[d]	29·3 ± 2·4
$IL\text{-}12^+$ basal	2·1 ± 0·5	1·1 ± 0·2[d]	1·2 ± 0·2	0·8 ± 0·1
$IL\text{-}12^+$ stimulated	15·5 ± 1·9	9·6 ± 1·4[e]	11·5 ± 2·1	10·6 ± 2·4
DC $CD33^{high}$	0·4 ± 0·1	0·4 ± 0·1	0·4 ± 0·05	0·3 ± 0·05
TNF^+ basal	7·8 ± 2·5	1·9 ± 0·5[d]	5·0 ± 1·2	1·2 ± 0·2[e]
TNF^+ stimulated	46·5 ± 4·4[c]	42·5 ± 3·5	33·4 ± 4·0	37·1 ± 3·7
$IL\text{-}12^+$ basal	3·0 ± 0·8	1·4 ± 0·3	2·0 ± 0·4	1·0 ± 0·6[e]
$IL\text{-}12^+$ stimulated	22·6 ± 3·3	14·7 ± 2·1[d]	15·8 ± 2·1	9·7 ± 1·1
DC $CD33^{intermed}$	0·2 ± 0·03	0·2 ± 0·03	0·3 ± 0·03	0·5 ± 0·03
TNF^+ basal	5·4 ± 1·4[c]	1·5 ± 0·4[e]	2·2 ± 0·4	1·5 ± 0·3[d]
TNF^+ stimulated	24·8 ± 4·0	22·3 ± 3·9	25·1 ± 4·9	24·4 ± 3·4
$IL\text{-}12^+$ basal	3·3 ± 1·0	1·3 ± 0·3[e]	1·1 ± 0·4	0·6 ± 0·2
$IL\text{-}12^+$ stimulated	11·9 ± 2·5	7·7 ± 1·7[d]	7·4 ± 1·5	7·4 ± 1·5

s.e.= Standard error; [a]unstimulated PB culture (6h); [b]lipopolysaccharide + IFN-γ-stimulated PB culture (6 h); [c]concentration before treatment significantly different ($P < 0·05$) from that of controls; [d]change significantly different ($P < 0·05$) between IBD patients and controls; [e]significant change during treatment at significance concentration of 5% ($P < 0·05$); [f]significant change during treatment at significance concentration of 1% ($P < 0·01$).

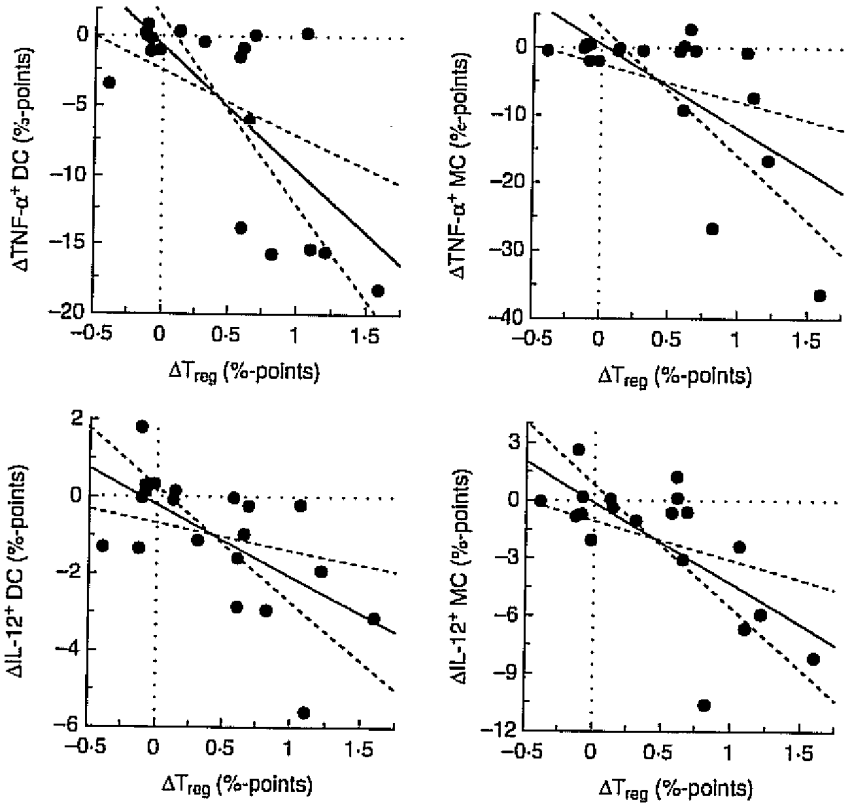

Fig. 4. Correlation between the changes in the percentage of putative regulatory T cells (T_{reg}, $CD4^+ CD25^{high}$) and in the percentage of tumour necrosis factor (TNF)-α- or interleukin (IL)-12-producing dendritic cells (DC) and monocytes (MC) in peripheral blood of inflammatory bowel disease (IBD) patients following treatment with the probiotic yogurt.

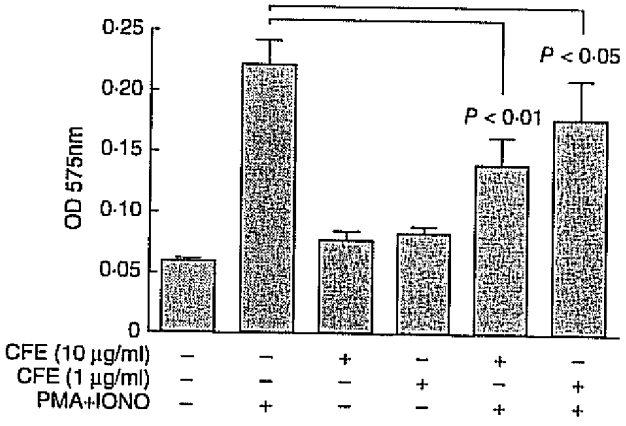

Fig. 5. Suppressive effect of cell-free extracts (CFE) of *Lactobacillus reuteri* RC-14 and *L. rhamnosus* GR-1 on the *in vitro* proliferative responses of peripheral blood mononuclear cells (PBMC). PBMC obtained from five healthy controls were cultured with (+) or without (–) PMA, ionomycin and CFE. Results are expressed as mean optical density (OD) at 575 nm +s.d., with higher OD corresponding to higher proliferation rate.

$CD4^+$ $CD25^{high}$ cells in the patients before and after treatment in the present study are consistent with the values reported by Maul and co-workers [27] for patients with active and inactive IBD, respectively.

The probiotic yogurt intake was associated with a number of potentially anti-inflammatory changes that are in harmony with the putative immunosuppressive role of the expanded $CD4^+$ $CD25^{high}$ cell population. The treatment was followed by a decrease in serum IL-12 concentration and a decreased percentage of TNF-α- and IL-12-producing monocytes and myeloid DC. Monocyte-produced TNF-α is one of the central final mediators in the inflammatory cascade of IBD. Its aetiological significance is well demonstrated by the success of TNF-α antibody treatments in inducing and maintaining remission [28]. IL-12 is the primary cytokine in directing T cell differentiation towards Th1 effector cells and thus is considered to be among the major cytokines in the pathogenesis of Crohn's disease [9]. An indirect correlation was observed between production of TNF-α and IL-12 by monocytes and DC and the numbers of $CD4^+$ $CD25^{high}$ cells in IBD patients. The causal relationship in such a correlation may be bidirectional. On one hand, low numbers of IL-12-producing monocytes and DC may indicate the expansion of an immature population of these potential antigen-presenting cells, DC in particular, which may then direct the T cell differentiation towards T_{reg} cells [29]. There is some indication that this would be a characteristic immunosuppressive response to Gram-positive commensal bacteria – an unlikely threat to the host [30]. On the other hand, established expansion of the T_{reg} cell population is likely to suppress the proinflammatory responses by monocytes and DC [31,32]. The increase in $CD4^+$ $CD25^{high}$ cells was also paralleled by reduced *ex vivo* production of IL-2 by T cells in response to polyclonal stimulus, a characteristic *in vitro* effect of $CD4^+$ $CD25^+$ T_{reg} cells [33]. Down-regulated T cell responsiveness was indicated further by reduced expression of the early T cell activation marker CD69 in response to *ex vivo* stimulus. However, the lack of influence on other T cell cytokines than IL-2 implies that the treatment did not have any considerable influence on the peripheral Th1/Th2 balance.

The data with unsupplemented yogurt indicate that the anti-inflammatory effects seen in the current study were dependent upon the presence of the *Lactobacillus* probiotic strains GR-1 and RC-14. The immunosuppressive capacity of these strains is supported by the finding that CFE of GR-1 and RC-14 inhibited the proliferative response of PBMC to polyclonal stimulus *in vitro*. Notably, the anti-inflammatory effects seen here are also consistent with previous *in vitro* data from our group, indicating that spent media from GR-1 culture inhibited the production of proinflammatory cytokines, including TNF and IL-12, with no effect on IL-10 production by murine macrophages exposed to *E. coli* or LPS [34].

The ability to expand the peripheral pool of $CD4^+$ $CD25^+$ T_{reg} cells could be beneficial in a wide variety of applications: in addition to the presumable ability to prevent and treat IBD, as suggested by animal studies [5,35], these cells can prevent and limit reactions to allergens, inhibit organ transplant rejections [36,37] and prevent autoimmune diseases, including arthritis [38] and insulitis as well as autoimmune thyroiditis and gastritis [39,40]. Further studies will determine whether or not significant expansion of the

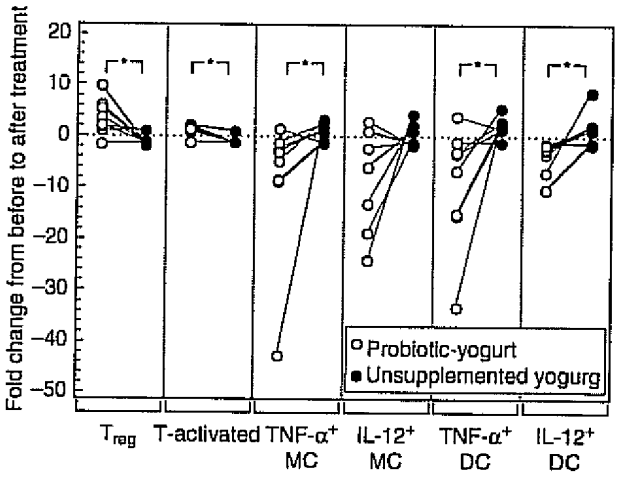

Fig. 6. Comparison of fold changes in the numbers of regulatory T cells (T_{reg}, $CD4^+$ $CD25^{high}$), activated T cells ($CD4^+$ $CD25^+$) and tumour necrosis factor (TNF)-α- and interleukin (IL)-12-producing monocytes (MC) and dendritic cells (DC) in inflammatory bowel disease (IBD) patients following treatment with probiotic yogurt or unsupplemented yogurt. Individuals are indicated by connective lines. *Change following treatment with probiotic yogurt significantly different from change following treatment with unsupplemented yogurt ($P < 0.05$).

$CD4^+$ $CD25^{high}$ cell population, associated with the intake of probiotic yogurt, is evident only in IBD patients. In the current study, only a minor expansion was seen in healthy controls. Such a difference to IBD patients is in agreement with previous studies indicating that probiotic therapies have commonly distinct effects on subjects with healthy *versus* inflamed mucosa [41]. Overall, fewer and generally more moderate changes in immunological parameters were observed in healthy subjects in this study. The effects were, however, in line with the anti-inflammatory effects seen in IBD patients, including a decrease in basal concentrations of IL-12- and TNF-α-producing monocytes and myeloid DC as well as a decrease in serum TNF-α concentrations. In contrast, faecal TNF-α concentration increased during the treatment, a reminder that peripheral effects and local effects in the intestine can be dramatically different.

Conclusion

Short-term consumption of yogurt supplemented with *Lactobacillus* strains GR-1 and RC-14 promoted the formation of a desirable anti-inflammatory environment in the peripheral blood of IBD patients, and showed no harmful effects in these patients or control subjects. This effect was associated with an increase in the presence of $CD4^+$ $CD25^{high}$ cells, a putative population of $CD4^+$ $CD25^+$ T_{reg} cells. Further clinical studies are now warranted to confirm the immunosuppressive functions and assess whether these peripheral effects are reflective of beneficial anti-inflammatory action locally in the intestine, resulting in clinical benefits such as prolonged remission of IBD.

In terms of the potential application of this nutritional supplement to patients with underlying complaints, such as HIV/AIDS patients with chronic diarrhoea or undernourished adults and children in developing countries, the findings supported at least acceptability of the taste and texture of the yogurt [42,43], and its safe use for 1 month. A preliminary study of its use by HIC/AIDS patients with chronic diarrhoea in Nigeria has shown that cessation of the diarrhoea occurs within 2 days of its use [44].

In short, probiotic yogurt can have nutritional as well as beneficial immune modulatory effects in patients with serious underlying disease.

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EXHIBIT G

Yogurt Containing Probiotic *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 Helps Resolve Moderate Diarrhea and Increases CD4 Count in HIV/AIDS Patients

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Abstract: HIV/AIDS is changing the human landscape in sub-Saharan Africa. Relatively few patients receive antiretroviral therapy, and many suffer from debilitating diarrhea that affects their quality of life. Given the track record of probiotics to alleviate diarrhea, conventional yogurt fermented with *Lactobacillus delbrueckii var bulgaricus* and *Streptococcus thermophilus* was supplemented with probiotic *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14. Twenty-four HIV/AIDS adult female patients (18 to 44 y) with clinical signs of moderate diarrhea, CD4 counts over 200, and not receiving antiretrovirals or dietary supplements, consumed either 100 mL supplemented or unsupplemented yogurt per day for 15 days. Hematologic profiles, CD4 cell counts, and quality of life was evaluated at baseline, 15 and 30 days postprobiotic-yogurt feeding. There was no significant alteration in the hematologic parameters of both groups before and after the probiotic-yogurt feeding. The probiotic yogurt group at baseline, 15 and 30 days had a mean WBC count of $5.8 \pm 0.76 \times 10^9$/L, $6.0 \pm 1.02 \times 10^9$/L, and $5.4 \pm 0.14 \times 10^9$/L, respectively. However, the mean CD4 cell count remained the same or increased at 15 and 30 days in 11/12 probiotic-treated subjects compared to 3/12 in the control. Diarrhea, flatulence, and nausea resolved in 12/12 probiotic-treated subjects within 2 days, compared to 2/12 receiving yogurt for 15 days. This is the first study to show the benefits of probiotic yogurt on quality of life of women in Nigeria with HIV/AIDS, and suggests that perhaps a simple fermented food can provide some relief in the management of the AIDS epidemic in Africa.

Key Words: probiotic, yogurt, HIV/AIDS, diarrhea, CD4

(*J Clin Gastroenterol* 2008;00:000–000)

The use of probiotics to alleviate gastrointestinal maladies, in particular diarrhea, has been well documented.[1] Fermented milk products, such as yogurt, have been shown to play a role in modulating the immune system.[2] In sub-Saharan Africa, fermentation of foods has become less commonplace over the past 50 years, in part due to importation of "Western" food practices. This region, already mired in poverty and malnutrition, is now engulfed by an HIV/AIDS epidemic that is crippling economies and causing relentless suffering. Women are particularly at risk, and 75% of new infections are believed to occur among young women and girls. Less than 0.1% of HIV/AIDS patients gain access to the highly active antiretroviral therapy they require, and up to 90% suffer from diarrhea.[3]

The CD4 count has become the conventional method of assessing the immune status of HIV-infected patients. Decisions regarding the initiation and determination of antiretroviral efficacy should be guided by monitoring the plasma HIV-RNA (Viral load) and CD4 counts. These parameters give the physician important information about the virologic and immunologic status of the patient and the risk of disease progression from HIV infection to the development of AIDS. With this CD4 decline comes increased risk of complications, especially potentially lethal opportunistic infections. In a study of 974 South African HIV/AIDS patients, those with a CD4 count between 201 to 350 cells/μL declined 20.5 cells/μL per year (0.8 per 2 wk), those between 351 and 500 fell 30.6 cells/μL per year (or 1.3 every 2 wk) and those greater than 500 cells/μL per year fell 47.1 cells/μL (1.8 per 2 wk).[4]

To date, there have been no studies on the use of probiotic yogurt for the management of diarrhea in HIV/AIDS patients, nor for halting the drop in CD4 counts. *Lactobacillus reuteri* has been shown to be safely

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Drs Anukam, Osazuwa and Osadolor have no conflicts.
Dr Reid declares he holds patents for the use of Lactobacillus GR-1 and RC-14 for urogenital health, but not for HIV diarrhea or CD4 applications.
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administered to HIV/AIDS subjects,[5] and to prophylactically benefit individuals susceptible to cryptosporidiosis.[6] The aim of the present study was to determine, in 40 HIV/AIDS-infected women, whether the addition of probiotic strains, *L. reuteri* (formerly *fermentum*) RC-14 with *L. rhamnosus* GR-1 to yogurt, could clear diarrhea and halt the drop in CD4 counts, of subjects with a starting CD4 count between 206 and 520 cells/μL.

MATERIALS AND METHODS

Subjects and Recruitment

Premenopausal women, attending the HIV Voluntary Counseling Test center in Benin City were recruited. The ethical review committee of the Faculty of Pharmacy, University of Benin, gave approval for the study. All testing was voluntary and included precounseling and postcounseling by trained HIV counselors. Each of the 24 subjects consented to the study after thorough explanation by the HIV counselor. All participants who were HIV positive were counseled and informed of their HIV status. Blood samples (4 mL) were collected into plain bottles for HIV antibodies, detected using Capillus HIV test kits (Cambridge). The reactive samples were further confirmed using the western blot technique (Biorad).

Inclusion Criteria, Study Subjects, Randomization, and Blinding

The inclusion criteria was for females aged 18 years and above; laboratory evidence of HIV infection; history of no previous antiretroviral therapy; and CD4 cell counts above 200 cells/μL. This value was chosen based on the recent study[7] showing the mean CD4 lymphocyte count of healthy Nigerians to be 685 ± 99, [95% confidence interval (CI) 487-883]. The value was twice that of HIV-infected subjects without AIDS-defining illness (314 ± 45, 95% CI 224-404). The CD4 lymphocyte count of HIV-infected with AIDS-defining illness was one-fourth or less (148 ± 30, 95% CI 88-208). The patients who were confirmed positive for HIV were invited to participate in the probiotic yogurt study. Lactose intolerance was an exclusion criterion, and none of the subjects enrolled suffered from this condition. Subjects were excluded if they were taking dietary supplements or any anti-retroviral therapies. It was not possible to control the dietary intake of the subjects, but interviews with each did not reveal any significant differences in the types of food consumed. The 24 HIV/AIDS adult female subjects enrolled were aged 18 to 44 years. The probiotic and placebo products were prepared by an independent laboratory and blinded to the physicians recruiting the patients and the researchers analyzing the data. Upon completion of the data analysis at 3 months, the code was broken.

Probiotic Yogurt

Conventional yogurts fermented with starter cultures of *Lactobacillus delbruekii* subsp. *bulgaricus* and *Streptococcus thermophilus* were supplemented with probiotic *L. rhamnosus*, GR-1 and *L. reuteri* RC-14. Ten milliliters of probiotic preparations (2.5×10^9 CFU/mL) were added to 990 mL of the fermented yogurt. The yogurt given to the subjects for the 15 days, maintained viable counts of both probiotics at 10^7/mL, as checked by culture. Both probiotic strains survive and grow well in milk and survive intestinal passage.[8] Twelve HIV/AIDS adult female subjects with clinical signs of moderate diarrhea each consumed 100 mL probiotic yogurt per day for 15 days. For the control group, 12 age-matched HIV/AIDS female subjects consumed 100 mL of unsupplemented yogurt. Each subject had 4 mL of venous blood collected into ethylenediaminetetraacetic acid containers, before study commencement and at 15 and 30 days follow-up. The samples were tested for hematologic parameters and CD4 cell counts using Dynabead technique (Dynal A.S., Oslo, Norway) which is the adopted technique for CD4 estimation under the national ARV programme in Nigeria.[9] The CD4 lymphocyte counts were expressed as cells/μL of blood.

Determination of CD4 Cell Counts

For each test, 155 μL of freshly obtained ethylenediaminetetraacetic acid anticoagulated blood was added to 350 μL of phosphate-buffered saline; 25 μL of suspended magnetic beads coated with anti-CD4 monoclonal antibody was added and the mixture incubated for 10 minutes at room temperature on a dynal mechanical rotator, to deplete blood of monocytes. The beads were separated using the magnetic particle concentrator and washed twice with phosphate-buffered saline. After addition of 50 μL lysing solution, cells were stained with 50 μL of Turks fluid and layered on a Neubauer counting chamber. The nuclei were integrally enumerated under light microscopy. Results were expressed as number of positive cells counted per microliter of whole blood.

Testing the Hematologic Parameters

Twelve hematologic parameters were determined at baseline, 15 and 30 days posttreatment, using autohematologic analyzer, CELL-DYN 1200 by Abbort, Inc, USA [white blood cells, red blood cells, hemoglobin, hematocrit (pack cell volume), mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, red blood cell distribution width, platelets].

Measuring Quality of Life at Baseline, 15 Days, 30 Days, and 3 Months

A structured questionnaire was designed to obtain clinical history and quality of life for each patient. The participants were asked to indicate any gastrointestinal discomfort particularly diarrhea which was described as Absent, Moderate (daily episodes of watery stools, mild nausea, and mild flatulence), or Severe (persistent watery stools, urgency and frequency of defecation, nausea and flatulence), and the presence of any opportunistic infections or skin conditions before and after the probiotic yogurt administration.

Statistics

Data are presented as the mean ± standard deviation, and n represents the number of participants from probiotic GR-1/RC-14 group and placebo group. Comparisons were made by use of the Student *t* test. Differences were regarded significant between the treatment groups with a P value less than 0.05.

RESULTS

There was 100% compliance at day 15, as determined by return for follow-up and inspection of empty yogurt containers. One subject's blood sample was not able to be scored due to technical error. No bacteremia was detected in any subject. Two subjects failed to show for the 30-day follow-up, despite being contacted. There was no significant alteration in the hematologic parameters (Table 1) of both groups after the treatment. The qualitative analysis of the urine parameters (color, bilirubin, urobilinogen, proteins, ketones, nitrite, glucose, and blood) did not change significantly in either group. However, urine leukocyte esterase, motile bacteria and white blood cells per high power field were significantly reduced in the probiotic yogurt group compared with the control group.

There were significant differences between the groups with respect to their CD4 cell counts (Table 2). Eight of 11 subjects at day 15 and 30, consuming unsupplemented yogurt had a drop in CD4 cell count, and the group's average drop coincided with the anticipated values predicted by Holmes et al.[4] On the contrary, 8/12 subjects at day 15 and 10/11 at day 30, who consumed the probiotic yogurt, had an increase in CD4 cell count. On average, this resulted in a 3 to 4 fold improvement compared to controls, and this was statistically significant ($P < 0.02$). No deaths occurred during the study or 3-month follow-up.

The subjects treated with the probiotic supplemented yogurt had rapid (within 2 d) resolution of their gastrointestinal discomfort, namely diarrhea with flatulence and nausea, compared to controls, and this remained so for the duration of treatment as well as during longer term follow-up (Table 3). Three of the placebo subjects developed skin rashes at 3 months, whereas none were noted in the probiotic-treated group.

DISCUSSION

This is the first study to show that probiotic supplemented yogurt, unlike regular yogurt, quickly alleviates diarrhea in HIV/AIDS patients, and has a positive outcome on CD4 counts. This effect occurred within days of consumption. The results obtained in this 24-subject study, provide strong evidence for a larger examination of the use of basic foods for management of the AIDS crisis, particularly in populations without access to antiretrovirals or expensive therapies. In a separate project in a poor area of Tanzania, yogurt supplemented with probiotic *L. rhamnosus* GR-1 is being made by mothers in a community kitchen. This project, Western Heads East (www.westernheadseast.ca) demonstrates the ability to reach poor people and have them engaged in a not-for-profit activity which can have important health outcomes.

Several patients had relatively good increases in their CD4 counts, even 15 days after completion of the probiotic yogurt intake, whereas one subject had a significant decrease in her CD4 count in the control group. The clinical significance and long term effects remain to be investigated.

Diarrhea is not only a problem with HIV/AIDS patients per se,[10] but it is one of the adverse effects of antiretroviral therapy.[11] The fact that a food, easily produced in developing countries, and indeed part of

TABLE 1. Mean Hematologic Profiles of HIV Patients at Baseline, 15 and 30 d Feeding of Yogurt Containing Probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 or Placebo

Hematologic Parameters	Probiotic Group at Baseline	Probiotic Days 15 and 30		Yogurt Group at Baseline	Yogurt Days 15 and 30		Statistical Analysis*	
	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30	Day 0	Day 30
TWBC × $10^3/\mu L$	5.8 ± 0.76	6.0 ± 1.02	5.4 ± 0.14	5.7 ± 2.6	5.6 ± 0.84	5.7 ± 1.9	NS	NS
Neutrophils %	62.0 ± 2.96	61 ± 0.85	51 ± 2.13	58.2 ± 1.4	51.4 ± 0.9	49 ± 2.6	NS	NS
Lymphocytes %	33.1 ± 2.40	33.0 ± 1.8	34 ± 1.45	34.3 ± 0.2	36.7 ± 0.2	41 ± 3.4	NS	0.05
MEB %	8.0 ± 1.35	7.7 ± 1.94	7.5 ± 2.17	8.0 ± 0.5	8.2 ± 1.24	9.0 ± 0.4	NS	NS
RBC × $10^6/\mu L$	4.76 ± 0.40	4.84 ± 0.5	5.1 ± 0.87	4.5 ± 0.8	4.4 ± 0.76	4.6 ± 0.7	NS	NS
Hb (g/dL)	12.6 ± 1.38	13.2 ± 1.2	13.8 ± 2.2	12.8 ± 1.6	13.1 ± 1.8	13.4 ± 2.3	NS	NS
HCT (PCV) %	38 ± 2.93	38 ± 2.74	39.1 ± 3.5	37.2 ± 3.9	37.7 ± 2.8	38.1 ± 3.5	NS	NS
MCV	89 ± 2.0	90 ± 1.8	91 ± 2.0	92 ± 1.2	92 ± 1.1	92 ± 1.0	NS	NS
MCH	31 ± 1	31 ± 1.2	31 ± 1	32 ± 0.3	32 ± 0.5	33 ± 0.3	NS	NS
MCHC	34 ± 0.12	33 ± 0.19	34 ± 0.14	34 ± 0.16	33 ± 0.8	33 ± 0.2	NS	NS
RDW	13 ± 0.1	13 ± 0.1	13 ± 0.2	13 ± 0.3	13 ± 0.2	13 ± 0.1	NS	NS
Platelet × $10^3/\mu L$	218 ± 14	220 ± 23	243 ± 25	210 ± 18	208 ± 26	201 ± 28	NS	NS

*Statistical analysis = level of significance for change from baseline and probiotic yogurt and placebo to day 30.
MEB indicates monocytes, eosinophils, basophils.
MCH indicates mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; PCV, pack cell volume; RDW, red blood cell distribution width.

TABLE 2. CD4 Cell Count of HIV Subjects who Consumed Unsupplemented or Probiotic Yogurt Containing *Lactobacillus* GR-1 and RC-14 for 15 Days

Probiotic Subjects at Baseline	Baseline CD4 Counts	Expected Change* at 15 d	Actual Change at 15 d	Expected Change* at 30 d	Actual Change at 30 d
1	367	−1.3	+9	−2.6	+14
2	331	−1.3	−1	−2.6	+33
3	298	−1.3	+13	−2.6	+3
4	305	−1.3	+8	−2.6	+3
5	326	−1.3	+7	−2.6	+8
6	401	−1.3	−3	−2.6	ND
7	269	−1.3	+5	−2.6	+1
8	394	−1.3	−2	−2.6	+2
9	520	−1.3	−2	−2.6	0
10	430	−1.3	+2	−2.6	+1
11	302	−1.3	+4	−2.6	+4
12	376	−1.3	+6	−2.6	+8
Mean ± SD CD4	359.9 ± 70.1	−1.3	+3.8	−2.6	+6.7
Placebo subjects at baseline					
13	401	−1.3	−4	−2.6	−4
14	398	−1.3	+1	−2.6	+2
15	297	−1.3	−11	−2.6	−13
16	376	−1.3	−2	−2.6	−4
17	341	−1.3	−1	−2.6	−1
18	342	−1.3	ND	−2.6	+4
19	206	−1.3	0	−2.6	−1
20	424	−1.3	−2	−2.6	ND
21	364	−1.3	−1	−2.6	−2
22	273	−1.3	−1	−2.6	−2
23	480	−1.3	−1	−2.6	−4
24	265	−1.3	0	−2.6	+1
	347.25 ± 76.81	−1.3	−2.0	−2.6	−2.2

Subjects were retested at 30 d.
ND indicates not done.

the heritage of many countries, can alleviate diarrhea, represents a significant potential means of reducing some deaths among HIV/AIDS patients. Having stated that, a larger study is needed to examine the use of probiotic yogurt in patients with lower CD4 counts and more severe diarrhea. The effects seemed to carryover for several months after eradication of the diarrhea. Given the apparent success of adding micronutrients to the diet of HIV/AIDS patients, albeit in those receiving antiretroviral drugs,[12] it would be worth investigating the effects of probiotics with micronutrients supplemented into yogurt in subjects who do not have access to highly active antiretroviral therapy therapy.

No side effects were noted, although 3 control subjects developed skin rashes by the third-month follow-up. This is not uncommon in patients developing end stage AIDS. Again, the extent to which probiotics can delay this event, seems worthy of investigation.

TABLE 3. Results of Quality of Life Parameters

	Diarrhea (With Flatulence and Nausea)			
	Absent	Moderate	Severe	Opportunistic Infections Such as Boils, Rashes, etc.
Probiotic Yogurt Group at Baseline (N = 12)	0	12 (100%)	0	0
2 d	12/12 (100%)	0	0	0
15 d	12/12 (100%)	0	0	0
30 d	7/11 (64%)	4/11 (36%)	0	0
3 mo	8/12 (67%)	4/12 (33%)	0	0
Yogurt Group at Baseline (N = 12)	0	12 (100%)	0	0
2 d	0/12	12/12 (100%)	0	0
15 d	1/11 (9%)	10/11 (91%)	0	0
30 d	3/11 (27%)	8/11 (73%)	0	0
3 mo	2/12 (17%)	10/12 (83%)	0	3 (mild rashes)

The 2-d and 3-mo values were ascertained from each subject by personal contact, outside of the 2 follow-up visits on days 15 and 30.

This study exemplifies the need to bring probiotics from the Northern and Western worlds to Africa, as well as to people in all countries who cannot afford pharmaceutical remedies, as recommended by the FAO.[13] If such remedies only provide temporary relief from this fatal disease, their value will be immense.

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EXHIBIT H

High incidence of pulmonary bacterial co-infection in children with severe respiratory syncytial virus (RSV) bronchiolitis

K Thorburn, S Harigopal, V Reddy, N Taylor and H K F van Saene

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RESPIRATORY INFECTION

High incidence of pulmonary bacterial co-infection in children with severe respiratory syncytial virus (RSV) bronchiolitis

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Thorax 2006;61:611–615. doi: 10.1136/thx.2005.048397

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Background: Respiratory syncytial virus (RSV) is the most common cause of viral lower respiratory tract infections (LRTI). Viral LRTI is a risk factor for bacterial superinfection, having an escalating incidence with increasing severity of respiratory illness. A study was undertaken to determine the incidence of pulmonary bacterial co-infection in infants and children with severe RSV bronchiolitis, using paediatric intensive care unit (PICU) admission as a surrogate marker of severity, and to study the impact of the co-infection on morbidity and mortality.

Methods: A prospective microbiological analysis was made of lower airways secretions on all RSV positive bronchiolitis patients on admission to the PICU during three consecutive RSV seasons.

Results: One hundred and sixty five children (median age 1.6 months, IQR 0.5–4.6) admitted to the PICU with RSV bronchiolitis were enrolled in the study. Seventy (42.4%) had lower airway secretions positive for bacteria: 36 (21.8%) were co-infected and 34 (20.6%) had low bacterial growth/possible co-infection. All were mechanically ventilated (median 5.0 days, IQR 3.0–7.3). Those with bacterial co-infection required ventilatory support for longer than those with only RSV ($p<0.01$). White cell count, neutrophil count, and C-reactive protein did not differentiate between the groups. Seventy four children (45%) received antibiotics prior to intubation. Sex, co-morbidity, origin, prior antibiotics, time on preceding antibiotics, admission oxygen, and ventilation index were not predictive of positive bacterial cultures. There were 12 deaths (6.6%), five of which were related to RSV.

Conclusions: Up to 40% of children with severe RSV bronchiolitis requiring admission to the PICU were infected with bacteria in their lower airways and were at increased risk for bacterial pneumonia.

Respiratory syncytial virus (RSV) is the most important viral cause for lower respiratory infection in infants and young children throughout the world.[1] It is one of the commonest causes of respiratory tract infection leading to respiratory failure. It has been estimated that in each year 600 000 deaths occur worldwide that are directly or indirectly attributable to RSV.[1] Factors that increase susceptibility to the virus include chronological age less than 6 weeks, bronchopulmonary dysplasia, congenital heart disease, prematurity, and immunodeficiency.[2–5] Although the mortality rate for those admitted to hospital may be as low as 1–3%, it increases in those with severe bronchiolitis requiring intensive care management.[2–6] In developed countries about 2% of infants and children admitted to hospital with RSV require assisted ventilation.[7] RSV bronchiolitis is a common cause for admission to a paediatric intensive care unit (PICU) in the winter season.[5–7]

The pharmacological management of RSV bronchiolitis, other than the use of supplementary oxygen, has long been debated.[8 9] In particular, many advocate against the routine use of antibiotics in bronchiolitis because of a reported low incidence of concurrent or secondary bacterial infections in patients with RSV.[10–17] However, these studies focused on extrapulmonary bacterial co-infection and included only limited numbers of children with severe respiratory compromise/failure.

Physiologically, the lower airways are normally sterile. Nevertheless, the relationship between bacterial co-infection and viral respiratory disease has previously been recognised, having an escalating incidence with increasing severity of respiratory illness.[18] Three retrospective studies investigated the occurrence of bacterial co-infection in children with severe RSV infection requiring PICU admission and found the incidence of pulmonary bacterial co-infection to vary between 17.5% and 44%.[19–21] In this study we prospectively investigated the incidence of pulmonary bacterial co-infection using established quantitative microbiology[22] in patients with severe RSV bronchiolitis on admission to a tertiary PICU, and evaluated the impact of the bacterial co-infection on morbidity and mortality.

METHODS

The study group comprised children admitted to the PICU at the Royal Liverpool Children's Hospital, a university affiliated multidisciplinary regional referral centre. The PICU is a 20-bed facility with an annual admission rate of over 1000 children. The overall mortality rate is 4.5%, with a predicted mortality of 6.25% using the paediatric index of mortality[23] and a standardised mortality rate of 0.72.

The main objectives of the study were (1) to determine the incidence of pulmonary bacterial co-infection in patients requiring admission to the PICU for severe RSV bronchiolitis; and (2) to study the impact of the co-infection on morbidity (including length of ventilation and inflammation) and mortality.

Children with RSV bronchiolitis, confirmed by RSV antigen testing and/or culture, requiring PICU admission and from whom lower airway secretions were obtained on admission were included in the study. Those with nosocomial RSV infections were excluded. Data were collected prospectively

Abbreviations: BAL, bronchoalveolar lavage; LRTI, lower respiratory tract infection; PICU, paediatric intensive care unit; RSV, respiratory syncytial virus

during three consecutive RSV seasons (winter) between 2002 and 2005 from RSV positive children admitted to the PICU.

The study was approved by the institutional ethics review board.

Respiratory support

Intubation was performed by our PICU retrieval team at the referring hospital, in our accident & emergency (A&E) department, or in one of the hospital wards prior to PICU admission. Alternatively, the anaesthetic team of the referring hospital intubated some of the patients before the arrival of the PICU retrieval team. It is policy that all children who require intensive care and ventilatory support are moved to the regional PICU.

The timing of extubation was judged clinically and not influenced by bronchoalveolar lavage (BAL) results.

Microbiological sampling

Diagnostic samples of nasopharyngeal aspirates (for RSV detection) and lower airway secretions (for bacterial culture) through endotracheal tube using sterile precautions[24] were taken on admission and processed immediately in the laboratory. Prior to routine bronchial toilet, a sterile suction catheter was passed down the endotracheal tube. Two 1 ml/kg aliquots of sterile 0.9% saline were instilled through the suction catheter, immediately followed by aspiration with constant pressure into a mucus trap. Samples were collected by specialist respiratory physiotherapists or PICU staff members. BAL was performed immediately after endotracheal intubation in children intubated in the hospital and on arrival in the PICU, and generally within 3 hours of endotracheal intubation for those admitted from other hospitals. All children within the region are only ventilated in the regional PICU, so are rapidly transferred to the PICU.

Surveillance samples of throat and rectum were obtained on admission and then twice weekly, in keeping with the routine surveillance practice in our unit.

Laboratory procedures

Viral

Nasopharyngeal aspirates were tested by the Directigen RSV test (Becton Dickinson Microbiology Systems, Maryland, USA). This is an in vitro enzyme immunoassay (ELISA) membrane test for the rapid and qualitative detection of RSV antigen directly from nasopharyngeal specimens. All samples negative for RSV using the ELISA membrane test were cultured using standard virological techniques at the Health Protection Agency.

Bacterial/yeast

Diagnostic or clinical samples were processed immediately in a qualitative and semi-quantitative way using standard microbiological methods. For all types of samples, macroscopically distinct colonies were isolated in pure culture. Standard methods for identification, typing, and sensitivity patterns were used for all micro-organisms.[25]

Antibiotic treatment

Patients with signs of infection received intravenous cefotaxime (150 mg/kg/day four times daily for up to 7 days) as first line treatment for 48 hours while awaiting culture results. Clinical status on presentation governed whether supplementary intravenous cover with an aminoglycoside (gentamicin 7.5 mg/kg/day three times daily for up to 7 days) was added. Antibiotics were rationalised once culture and sensitivity results became available.

Definitions

Bacteria positive: the presence of micro-organisms in the lower airways which is normally sterile.

Co-infection: Infection is a microbiologically proven, clinical diagnosis of inflammation, local and/or generalised. In this study clinical signs were unreliable as all patients had bronchiolitis, so microbiological definitions were used. Bacterial co-infection required bacteria colony counts $\geq 10^5$ cfu/ml of diagnostic sample for each single species obtained from lower airway secretions *and*, on a semi-quantitative scale of + = few ($<50\times10^6$/l), ++ = moderate ($>100\times10^6$/l), and +++ = many leucocytes ($>1000\times10^6$/l), the presence of at least a moderate (++) number of leucocytes.[26 28]

Low bacterial growth: Diagnostic samples from lower airway secretions which yielded $<10^5$ cfu/ml of diagnostic sample and the presence leucocytes.

The chest radiographic appearance was not used to diagnose bacterial co-infection as changes on the chest radiograph are not pathognomonic of secondary bacterial or viral infections.[18 29]

Analysis of data

Data were collected prospectively. Prediction of mortality using the paediatric index of mortality was obtained on the patient's first contact with the PICU team.[21] Results were expressed as a percentage of the total study population; median and interquartile ranges (IQR) were used to describe the demographic distributions.

Continuous data were analysed using the Wilcoxon-Mann-Whitney (W-M-W) test. Categorical data were analysed using Fisher's exact or McNemar's test. Correlation was assessed using Spearman's rank test (two tailed). Multivariate analysis was performed using linear and logistic regression analysis.

Statistical calculations were performed with the Statistical Program for Social Science release 11.0.0 (SPSS 11, Chicago, IL, USA). A p value of <0.05 was considered statistically significant.

RESULTS

A total of 181 children (103 boys and 78 girls) of median age 1.6 months (IQR 0.5–4.6) were admitted to the PICU with RSV positive bronchiolitis during the three consecutive RSV seasons (2002–5). The indication for PICU admission for these children was ventilatory/respiratory support (respiratory failure (n = 172) and/or life threatening apnoeas (n = 9)). All patients were mechanically ventilated for a median of 5.0 days (IQR 3.0–7.3). 165 children were enrolled in the study; an admission BAL sample was not available in 16 patients (8.8%).

The demographic characteristics, inflammatory markers, antibiotic history, and mortality of the RSV positive children in the subgroups RSV only, bacterial co-infection, low bacterial growth, and bacteria positive (co-infection + low bacterial growth) are shown in table 1. The white cell count, neutrophil count, and C-reactive protein (CRP) levels did not differ between the groups on admission or during days 1–5 in the PICU.

Although all patients were admitted primarily for respiratory disease, 43% (71/165) of them had other co-morbidities (congenital heart disease n = 37, chronic lung disease n = 8, immunodeficiencies n = 4, abnormality of large airways n = 5, congenital heart disease and abnormality of large airways n = 8, congenital heart disease and chronic lung disease n = 4, neuromuscular disease n = 7). Co-morbidity did not increase the risk of positive bacterial cultures (odds ratio 0.77, 95% CI 0.55 to 1.09).

Table 1 Patient characteristics according to culture result (n = 165)

	RSV only	Bacterial co-infection (>10^5 cfu/ml)	Low bacterial growth (<10^5 cfu/ml)	Bacteria positive (co-infection + low bacterial growth)
N (% of total)	95 (57.6%)	36 (21.8%)	34 (20.6%)	70 (42.4%)
Origin (retrieved/intra-hospital/A&E)¶	48/29/18	11/17/8	14/11/9	25/28/17
Age (months)	1.4 (0.4–3.9)	1.3 (0.7–2.5) p=0.8*	3.5 (1.2–10) p=0.04†	1.8 (0.9–4.6) p=0.9‡
Paediatric index of mortality	0.08 (0.03–0.12)	0.09 (0.04–0.14) p=0.6*	0.08 (0.06–0.12) p=0.5†	0.08 (0.05–0.13) p=0.6‡
Length of ventilation (days)	4 (3–7)	6 (4–8) p<0.01*	6 (5–9) p<0.01†	6 (4–8) p<0.01‡
Admission OI in PICU	8 (5–12)	6 (4–9) p=0.2*	9 (6–12) p=0.6†	7 (4–11) p=0.7‡
Admission VI in PICU	26 (18–39)	27 (16–44) p=0.9*	26 (20–32) p=0.2†	27 (19–39) p=0.8‡
White cell count (×10^9 cells/l) on PICU admission	9.8 (7.2–13.7)	10.6 (7.1–13.5) p=0.5*	11.5 (6.9–14.7) p=0.6†	11.3 (7.1–13.8) p=0.4‡
Neutrophil count (×10^9 cells/l) on PICU admission	5.2 (2.9–7.6)	7.1 (3.9–10.3) p=0.1*	5.8 (3–10.3) p=0.9†	6.2 (3.6–10.3) p=0.2‡
CRP (mg/l) on PICU admission	14 (4–45)	14 (5–52) p=0.9*	21 (4–46) p=0.9†	18 (4–49) p=0.9‡
Antibiotics before PICU admission	48%	36%	44%	40% p=0.08‡
Time on prior antibiotics (days)	1 (1–2)	1 (1–3)	1 (1–3)	1 (1–3)
Mortality (RSV related deaths)	8 (3)	2 (1)	2 (1)	4 (2)
Percentage with co-morbidities§	40%	61%	41%	51% p=0.6‡

Data shown as median (IQR).
cfu/ml, colony forming units of a single bacterial species per ml of diagnostic sample; OI, oxygen index (mean airways pressure (MAP) × FiO_2/PaO_2); VI, ventilation index (respiratory rate × $PaCO_2$ × peak inspiratory pressure/1000); CRP, C-reactive protein; PICU, paediatric intensive care unit.
*RSV only *v* bacterial co-infection.
†RSV only *v* low bacteria growth.
‡RSV only *v* all those positive for bacteria (bacterial co-infection + low bacteria growth).
¶Retrieved, patients retrieved from other hospitals; intra-hospital, patients admitted from wards within our hospital; A&E, patients admitted directly from the Accident & Emergency department.
§Co-morbidities = congenital heart disease, chronic lung disease, abnormality of large airways, immunodeficiencies, neuromuscular disease.
Wilcoxon-Mann-Whitney test used for comparisons except for prior antibiotics and co-morbidities (McNemar's test).

Overall, 45% (74/165) received antibiotics before admission to the PICU (that is, started by the referring hospital or ward), most often cefotaxime or ceftriaxone. The breakdown between the subgroups is shown in table 1. Receipt of antibiotics before PICU admission did not affect the paediatric index of mortality (p = 0.6, W-M-W test) and length of ventilation (p = 0.2, W-M-W test). All except eight patients were continued or commenced on antibiotics in the PICU (usually cefotaxime). Antibiotics were continued for a median of 5 days (IQR 3–6). The empirical use of antibiotics was at the discretion of the attending consultant.

Sex, age, paediatric index of mortality, co-morbidity, receipt of prior antibiotics, time on antibiotics before intubation, admission oxygen and ventilation index were not predictive of positive bacterial cultures by univariate or multivariate analysis (all p values >0.16).

Table 2 Bacterial isolates (n = 98) obtained on admission to the PICU from the lower airway in 70 children with severe RSV bronchiolitis

	Co-infection (>10^5 cfu/ml)	Low bacterial growth (<10^5 cfu/ml)
Community organisms[28 30]		
H influenzae	17	11
S aureus	10	12
M catarrhalis	8	10
S pneumoniae	6	6
S pyogenes	1	
Abnormal organisms[28 30a]		
P aeruginosa	4	3
B pertussis	1	
K pneumoniae	1	1
E coli	1	1
E cloacae and *C freundii*	1	
P mirabilis		1
S agalactiae		1
N meningitidis		1
MRSA		1

23 patients had multiple organisms (18 had two, 5 had three bacteria); community organisms were involved in 91% of these cases compared with 77% single isolates (p = 0.2, Fisher's exact test).
*67% (10/15) had chronic illnesses.

The organisms isolated from lower airway secretions obtained on admission are shown in table 2. All those with positive endotracheal bacteriological specimens had the same organisms isolated on admission surveillance swabs. Community organisms accounted for 83% (81/98) of the bacteria cultured.

There were 12 deaths (6.6%), five of which (2.8%) appeared to be RSV related as the patients were still RSV positive when they died. Two patients with leukaemia on chemotherapy died from RSV pneumonitis on days 1 and 16, respectively. Neither had proven bacterial co-infection and both received broad spectrum empirical antibiotic treatment. Other associated causes included singles cases of hypoplastic right heart coupled with cystic fibrosis (on day 8), *B pertussis* co-infection with hypoxaemic respiratory failure requiring extracorporeal membrane oxygenation (on day 26), and a child with a congenital myopathy (on day 8). The remaining seven deaths occurred 6–31 days after admission subsequent to the RSV cultures becoming negative. Causes of these RSV "unrelated" deaths included complex congenital heart disease (n = 3), multiple congenital anomalies (n = 2), congenital myopathy (n = 1), anoxic brain injury (n = 1). Positive bacterial cultures did not predict death (odds ratio 1.3, 95% CI 0.57 to 2.95), but co-morbidity did (odds ratio 0.51, 95% CI 0.37 to 0.7).

DISCUSSION

This observational study over three consecutive RSV seasons evaluating bacterial pulmonary co-infection found that 42% of children admitted with severe RSV infection harboured bacterial pathogens in their lower airways. These critically ill children run a serious risk of developing bacterial pneumonia.[10 11]

BAL samples were collected very soon after intubation so significant growth densities of bacteria reflect pathogens in the normally sterile lower airways. The high number of colony forming units makes it highly unlikely that the micro-organisms isolated were "pushed down" the trachea on intubation. We acknowledge that the number of leucocytes in lower airways secretions will also be influenced by RSV infection and therefore relied on bacterial growth. The microbiological criteria were strict and avoided potentially confounding clinical factors. This microbiological approach is supported by recent literature concerning ventilator associated pneumonia (VAP).[12 13] On the other hand, it must be appreciated that our study group was very different from this VAP group as they had "virgin" iatrogenically uncontaminated lower airways. Certainly, in the group with co-infection, substantial bacterial growth densities occurred far too soon after intubation to have been oropharyngeal flora transported there by the endotracheal tube. If anything, the strict microbiological criteria probably underestimated the number co-infected by categorising many of them as having low bacterial growth. We accept that differentiating the groups into "co-infected" and "low bacterial growth" may be somewhat artificial as the lower respiratory tract should be free from bacteria.

The term "co-infection" was used as, at the time of PICU admission, these infections could either be secondary or concurrent. It would not be easy to detect the "chicken" from the "egg" as far as which was primary—the RSV or the bacteria—although a viral infection destroying cilia is in general required for a bacterial co-infection.[18] The true co-infection rate is likely to be higher than the 22% rate detected, as 45% of the cases received antibiotics before admission to the PICU. These antibiotics may have converted some of the "co-infection" patients into the "low bacterial growth" group, or even prevented bacterial growth altogether.

Previous studies have examined bloodstream, otitis media, or urinary tract infections in children with bronchiolitis, very few of whom had severe RSV bronchiolitis requiring intensive care.[10–17] These studies generally found a very low incidence of secondary serious bacterial infection (1.2%)[14] or bacteraemia (0.6%)[16] in their hospitalised RSV patients. Because these studies did not specifically concentrate on those with severe bronchiolitis, it is difficult to extrapolate their results to this population. Duttweiler *et al* retrospectively studied 127 infants admitted to intensive care for RSV bronchiolitis and found that 25 (44%) of the 57 ventilated and endotracheally sampled infants had "concomitant bacteria pneumonia".[19] Similarly, the retrospective study of Kneyber *et al*[20] (82 PICU admissions with 65 (79%) ventilated) found that nine (33%) of the 24 children on whom admission endotracheal aspirates were performed had a positive bacterial culture. Randolph *et al*[21] retrospectively examined 165 previously healthy infants admitted to the intensive care unit over a 12 year period with laboratory confirmed RSV infection, 63 (38%) of whom required mechanical ventilation. They found that 17.5–38% of the 63 intubated infants had "probable" or "possible" bacterial pneumonia. The incidence of bacterial pulmonary infection in these retrospective PICU reports is in keeping with that of this prospective study in which all bronchiolitic admissions were included.

Fifty one percent of the patients with bacteria in their airways and 40% of the children with RSV only had co-morbidities (congenital heart disease, chronic lung disease, large airway abnormality, immunodeficiency, neuromuscular disease). This is in keeping with well recognised risk factors associated with more severe RSV disease.[4 5 9 10 14] Co-morbidities did not account for differences in length of ventilation between the study groups, but did contribute towards mortality. The high percentage with co-morbidities is most probably also influenced by the fact that our centre is the regional paediatric cardiac referral centre, which means that children with congenital heart disease and bronchiolitis are more likely to be referred to our PICU for intensive care management.

There were fewer deaths in the bacteria positive group than in those with RSV only. However, when adjusted for those children who had recovered from their RSV infection only to die later from RSV unrelated causes, both groups had similar mortalities (2.9% *v* 3.2%). The paediatric index of mortality is a point of first contact score that is used to assess the risk of death while in the PICU.[21] The paediatric index of mortality scores for all the groups were similar, suggesting that all groups had matching severity of illness on admission to the PICU. Yet those with positive bacterial cultures required ventilatory support for longer than those with RSV only. Kneyber *et al*[20] reported a similar finding. Although length of ventilation was significantly different between the groups, other respiratory support and inflammation indices did not differ between them (table 1). Perhaps the general inflammatory response once triggered by RSV is not so refined as to be further enhanced by concomitant bacterial infection. Others have also found inflammatory markers unhelpful in differentiating bacterial infection in this group of patients.[20 35 36] Unfortunately, we were unable to find any early clinical measurements which would identify which RSV patients had bacterial co-infection.

Receipt of prior antibiotics and length of time on them did not predispose to bacterial co-infection. Moreover, many of the children with RSV had received antibiotics for only one day or less (often a single dose close to intubation). The fact that nearly all the RSV positive children received antibiotics in our PICU limited any interpretation on the impact of antibiotics on their outcome. All those patients with positive bacteriology in their endotracheal secretions had the same organisms isolated on admission surveillance swabs, indicating primary endogenous infection.[37] This reinforces the view that potential pathogens are carried first in the nasopharynx and then there is migration down the trachea into the lower airways.[37 38] The organisms isolated on admission were generally normal community organisms because most of the patients were in good health before RSV infection and PICU admission.[37 39] *Pseudomonas aeruginosa* was the most common of the abnormal bacteria (table 2). All these patients were carriers of abnormal organisms in their throats, and in most the common denominator for their abnormal carriage was chronic illness.[37 39] Interestingly, *Streptococcus pneumoniae* was isolated from relatively few patients. This could be the result of prior antibiotic use.[40]

Although most LRTI in children are viral in aetiology, mixed viral/bacterial infections are seen in up to a quarter of hospitalised children.[41 42] In addition, there is a risk of developing bacterial superinfection with viral LTRI.[18] These issues have contributed to the recommendations by the World Health Organization that the treatment of community acquired pneumonia should include empirical antibiotics.[41 43] Concerns that using antibiotics (in our case cefotaxime) pre-emptively in this group of critically ill children would breed antibiotic resistance have been shown to be unfounded in a 4 year study.[44] Assessment of the influence of antibiotics on

children with severe bronchiolitis would require a prospective randomised controlled trial.

This study has shown that up to 40% of patients admitted with severe RSV bronchiolitis were infected with bacteria in their lower airways. Co-morbidity (congenital heart disease, chronic lung disease, large airway abnormality, immunodeficiency, neuromuscular disease) predisposes to more severe RSV disease.

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EXHIBIT I

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Novel beta-lactam antibiotics derivatives: their new applications as gene reporters, antitumor prodrugs and enzyme inhibitors.

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Since the antibiotic properties of penicillin were first noticed in the beginning of last century, beta-lactam based antibiotics have been well developed as miracle drugs for the therapy of bacterial infectious diseases in clinics. Recently, these "old" antibiotics and their relevant derivatives have also found new applications as gene reporters, anti-cancer prodrugs and enzyme inhibitors. In this review, we will introduce the latest developments in the study of these new applications based on literatures reported over the last decade. The first section covers the recent developments of beta-lactam antibiotics as drugs against bacteria, the second section briefly discusses the occurrence of bacterial resistance and mechanistic studies of beta-lactam resistance in bacteria, the third section presents the current development of fluorogenic cephalosporin based beta-lactam probes for real-time imaging of gene expression, and the fourth section describes relevant studies on beta-lactam based substrates as anti-tumor prodrugs. Beta-lactam substrates as protease inhibitors will be also described in the fifth section. The final section summarizes future perspectives for beta-lactam antibiotic derivatives as scaffolds in the fields of molecular imaging, drug delivery and enzymatic assays.

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EXHIBIT J

ORIGINAL ARTICLE

Molecular Identification of Bacteria Associated with Bacterial Vaginosis

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ABSTRACT

BACKGROUND

Bacterial vaginosis affects millions of women and is associated with several serious health conditions. The cause of bacterial vaginosis remains poorly understood despite numerous studies based on cultures. Bacteria in microbial communities can be identified without cultivation by characterizing their ribosomal DNA (rDNA) sequences.

METHODS

We identified bacteria in samples of vaginal fluid with a combination of broad-range polymerase-chain-reaction (PCR) amplification of 16S rDNA with clone analysis, bacterium-specific PCR assay of 16S rDNA, and fluorescence in situ hybridization (FISH) performed directly on vaginal fluid from 27 subjects with bacterial vaginosis and 46 without the condition. Twenty-one subjects were studied with the use of broad-range PCR of 16S rDNA, and 73 subjects were studied with the use of bacterium-specific PCR.

RESULTS

Women without bacterial vaginosis had 1 to 6 vaginal bacterial species (phylotypes) in each sample (mean, 3.3), as detected by broad-range PCR of 16S rDNA, and lactobacillus species were the predominant bacteria noted (83 to 100 percent of clones). Women with bacterial vaginosis had greater bacterial diversity ($P<0.001$), with 9 to 17 phylotypes (mean, 12.6) detected per sample and newly recognized species present in 32 to 89 percent of clones per sample library (mean, 58 percent). Thirty-five unique bacterial species were detected in the women with bacterial vaginosis, including several species with no close cultivated relatives. Bacterium-specific PCR assays showed that several bacteria that had not been previously described were highly prevalent in subjects with bacterial vaginosis but rare in healthy controls. FISH confirmed that newly recognized bacteria detected by PCR corresponded to specific bacterial morphotypes visible in vaginal fluid.

CONCLUSIONS

Women with bacterial vaginosis have complex vaginal infections with many newly recognized species, including three bacteria in the Clostridiales order that were highly specific for bacterial vaginosis.

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BACTERIAL VAGINOSIS IS A COMMON condition, affecting millions of women annually,[1] and is associated with numerous health problems including preterm labor resulting in low birth weight,[2,3] pelvic inflammatory disease,[4,5] and acquisition of the human immunodeficiency virus.[6,7] Malodorous vaginal discharge may be the only symptom of bacterial vaginosis, and many affected women are asymptomatic.[8]

Studies using cultivation methods have shown that women with bacterial vaginosis have loss of vaginal lactobacilli and concomitant overgrowth of anaerobic and facultative bacteria. Several bacteria have been implicated in bacterial vaginosis, such as *Gardnerella vaginalis*[9] and *Mobiluncus curtisii*,[10] but these species are also found in subjects who do not have bacterial vaginosis and thus are not specific markers for disease.[11] For this reason, bacterial cultivation of vaginal fluid has not proved useful for the diagnosis of bacterial vaginosis. Rather, clinical criteria or Gram's staining of vaginal fluid is used for diagnosis.

At least three of the following four elements must be present to fulfill the clinical criteria of Amsel et al. for bacterial vaginosis[12]: thin, homogeneous, milky vaginal discharge; vaginal-fluid pH greater than 4.5; a positive whiff test (i.e., production of a fishy odor when 10 percent potassium hydroxide is added to a slide containing vaginal-fluid); and clue cells (>20 percent of epithelial cells with adherent bacteria) on microscopical examination of vaginal fluid.[12] An alternative diagnostic approach is to use Gram's staining of vaginal fluid to distinguish normal vaginal flora (i.e., gram-positive rods and lactobacilli) from bacterial vaginosis flora (gram-negative morphotypes)[13] according to the Nugent score.[14]

Koch's postulates for establishing disease causation have not been fulfilled for any bacterium or group of bacteria associated with bacterial vaginosis. The disorder responds to treatment with antibiotics such as metronidazole and clindamycin, but metronidazole has a low level of activity in vitro against *G. vaginalis* and *M. curtisii*. Relapse and persistence are common.[11] Thus, the causes and pathogenesis of bacterial vaginosis remain poorly understood, and management can be challenging.

Only a small fraction of the bacteria present in most microbial ecosystems are amenable to propagation in the laboratory.[15] Bacteria in complex microbial communities can be identified by characterizing their ribosomal RNA genes (rDNA), an approach that has the advantage of detecting fastidious or cultivation-resistant organisms.[16] We sought to detect and identify bacteria in vaginal-fluid samples with the use of molecular methods.

METHODS

STUDY POPULATION

A total of 87 women were recruited from two groups known to have a high prevalence of bacterial vaginosis.[17-20] We studied the first 73 women enrolled. Fourteen were patients at Public Health–Seattle and King County Sexually Transmitted Disease (STD) Clinic, and 59 were study participants at Harborview Medical Center Women's Research Clinic, in Seattle, who reported engaging in same-sex behavior in the previous year and most of whom were also sexually active with male partners. Women were eligible if they were 16 to 45 years of age and able to provide written informed consent.

Single baseline samples of vaginal fluid from the 73 subjects were studied with the use of bacterium-specific polymerase-chain-reaction (PCR) assays of 16S rDNA, including samples from 27 subjects with bacterial vaginosis as defined according to the clinical criteria of Amsel et al.[12] and from 46 subjects without bacterial vaginosis. Samples of vaginal fluid from 21 of these 73 subjects were studied with the use of broad-range bacterial PCR of 16S rDNA with clone analysis, including single baseline samples from 9 subjects with bacterial vaginosis and 8 subjects without bacterial vaginosis and multiple follow-up samples from 4 subjects with newly diagnosed, resolved, relapsed, or persistent bacterial vaginosis (11 samples).

At both clinics, subjects underwent examinations with a speculum to collect vaginal fluid for saline microscopy, microscopical visualization with potassium hydroxide, measurement of pH, and assessment for an amine odor. Subjects at the STD clinic were tested routinely for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, and those seen at the Women's Research Clinic were tested with the use of standard screening and diagnostic criteria.[21] Vaginal fluid was collected for molecular studies by brushing the lateral vaginal wall with a foam swab that was subsequently frozen.

Written informed consent was obtained from all participants in this prospective study, which was approved by institutional review boards at the Fred Hutchinson Cancer Research Center and the University of Washington in Seattle. Samples

were collected between October 16, 2001, and May 5, 2004.

SAMPLE PREPARATION

Swabs for PCR assays were placed in 15-ml conical tubes with saline, and the mixture was vortexed to dislodge cells. Sham swabs (with no patient contact) were processed in parallel to monitor for contamination of reagents. Saline solution was centrifuged at 14,000×*g* for 10 minutes, and the supernatant was discarded. The pellet was digested with the use of the QIAmp stool kit (Qiagen) with a 95°C lysis step according to the manufacturer's instructions. Methods for broad-range PCR of 16S rDNA, bacterium-specific PCR, and fluorescence in situ hybridization (FISH) are described in the Supplementary Appendix (available with the full text of this article at www.nejm.org).

STATISTICAL ANALYSIS

The samples of vaginal fluid were assessed as they were obtained, and the decision to stop the analysis at 73 subjects was made on the basis of data showing that there were statistically significant associations between the detection of several bacterial species and bacterial vaginosis. Differences in the number of taxa detected in bacterial vaginosis and control libraries of clones were assessed with the use of the Mann–Whitney U test. Univariate associations between the detection of individual bacteria by bacterium-specific PCR and the presence of bacterial vaginosis were measured with Fisher's exact test and SPSS software (version 10.1.4), and exact confidence intervals were calculated with the use of Stata software (version 8.2). Multivariate logistic-regression analysis was performed with the use of LogXact software (version 4.0.2), and covariates, in addition to individual bacteria and combinations of bacteria according to bacterium-specific PCR included the age of the subject, the clinical site of enrollment, the presence or absence of a report of abnormal vaginal discharge, and the presence or absence of a report of having had sex with men. All tests for significance were two-sided, and P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Of the 73 enrolled women, 27 had bacterial vaginosis at baseline and 46 did not (see Table 1 of the Supplementary Appendix). Slightly more than half the women with bacterial vaginosis were symptomatic, and very few had another genitourinary infection in addition to bacterial vaginosis.

Broad-range bacterial PCR of 16S rDNA with analysis of cloned sequences was performed on 28 samples of vaginal fluid from 21 subjects. Table 1 shows the bacterial species (phylotypes) detected and the percentage of clones from each library derived from these bacteria. Among subjects for whom only single baseline samples were evaluated (Table 1), those without bacterial vaginosis had a mean of 3.3 bacterial phylotypes per library (range, 1 to 6). Lactobacillus species were the dominant bacteria detected (83 to 100 percent of clones per library; mean, 97 percent), particularly *L. crispatus* and *L. iners*. Most bacterial 16S rDNA sequences in subjects without bacterial vaginosis closely matched known bacteria.

Broad-range bacterial PCR analysis of 16S rDNA from the vaginal fluid of subjects with bacterial vaginosis showed a high level of species diversity (Table 1), with a mean of 12.6 bacterial phylotypes per library of clones (range, 9 to 17), a level significantly higher than that in subjects without bacterial vaginosis (P<0.001). Overall, newly recognized bacterial phylotypes (bacteria with 16S rDNA that had <98 percent similarity to known sequences) were present in 58 percent of clones per library derived from bacterial vaginosis samples (range, 32 to 89 percent).

L. crispatus 16S rDNA was not detected in libraries of clones from subjects with bacterial vaginosis, whereas *L. iners* was detected in most subjects. *G. vaginalis* was detected in all bacterial-vaginosis clone libraries and *M. mulieris* in one library. Mycoplasma species were not detected in any clone library, despite sequence homology with the broad-range PCR primers of 16S rDNA that were used. Other bacteria frequently detected in subjects with bacterial vaginosis included *Atopobium vaginae*, two megasphaera species, two distinct dialister phylotypes, *Leptotrichia amnionii* and the related bacterium *Sneathia sanguinegens*, *Porphyromonas asaccharolytica*, and a bacterium distantly related to *Eggerthella hongkongensis* (92 percent sequence similarity). Nine different bacteria related to prevotella species were detected. Three phylogenetic clusters of these bacteria were only distantly related to known prevotella species (<95 percent sequence similarity), and we have designated these clusters prevotella genogroups 1, 2, and 3 on the basis of shared sequences within each group. Less frequently detect-

Table 1. Bacteria Identified by Broad-Range 16S rDNA Polymerase Chain Reaction in Vaginal Fluid from Subjects with Bacterial Vaginosis and

Broad-Range 16S rDNA PCR Clones	Subjects with BV									Control Subjects without BV							
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8
																	percentage of clones
Lactobacillus crispatus										49	74	99	48	60	89	40	100
L. jensenii													2		1		
L. gallinarum										13							
L. gasseri											9			1			
L. vaginalis													2				
Staphylococcus epidermidis											2						
S. lugdunensis													1				
Clostridium perfringens (96%) AB045286														3			
Ureaplasma parvum											1						
L. iners				7	22	3		1	5	38		1	46	36	10	60	
Gardnerella vaginalis	35	13	2	4	28	1	25	31	39		14						
Uncultured AB034121 (90.6%) (BVAB1)		43	66	34		36	17		26								
Uncultured AF407407 (90.9%) (BVAB2)	10	4		10	3	5	4		4								
Uncultured ULO278163 (92.9%) (BVAB3)	1	1					1										
Atopobium vaginae	5		3	21	1	3	3	11	11								
Leptotrichia amnionii	6		2	8	1	3	10	10									
Megasphaera elsdenii (93.8%) AY038994	4	10	7	1	18	3	2	13	6								
M. micronuciformis (94.6%) AF473833							1										
Eggerthella hongkongensis (91.8%) AY288517	2	2	2	1	4	1	3	8									
Porphyromonas asaccharolytica				2		5											
Dialister species (α)	1	1	2	2									1				
Dialister species (β) (94.8%) AF371693		1	2			2	2	1									
Sneathia sanguinegens	3		2		16	9	9										
Prevotella genogroup 1	21	24		1	7	9	12	20									
Prevotella genogroup 2	7			4		6	7		1								
Prevotella genogroup 3			7					3									
P. bivia									6								
P. buccalis																	
P. dentalis (93.2%) X81876						2	2										
P. disiens																	
P. oulorum (90.6%) L16472																	
P. shahii (90.7%) AB108825																	
Uncultured 4C28d-23 (91.2%) AB034149	1		3	1		2											
Candidate division TM7 (93.7%) AF125206			2	2		8											
Mobiluncus mulieris																	
Peptoniphilus lacrimalis							1										
Peptoniphilus species		1				2											
Peptostreptococcus micros (97.8%) AF542231	3			2				1									
Gemella bergeriae (95.8%) Y13365	1																
Aerococcus species								1	2								
Anaerococcus tetradius																	
Uncultured (89.8%) AF371910																	
Uncultured (88.4%) AJ400235																	
Veillonella species							1										

* For sequences with less than 98 percent similarity to known 16S rDNA sequences, the percent similarity to the closest match in GenBank is from each bacterial 16S rDNA phylotype or species (rows) is displayed for each sample library (column). Single vaginal-fluid samples were tained from four subjects in a longitudinal study and these samples are labeled L1 through L4 to designate the four subjects, and a, b, or c to desig- between collection of the first sample and subsequent samples, and whether subjects had bacterial vaginosis at that visit (BV+) or did not in BV– subjects.

from Those without Bacterial Vaginosis.*

Clinical Status of Infection										
Incident			Cured		Relapsed			Persistent		
BV–	BV–	BV+	BV+	BV–	BV+	BV–	BV+	BV+	BV+	BV+
Day 0	Day 28	Day 52	Day 0	Day 30	Day 0	Day 28	Day 100	Day 0	Day 34	Day 64
L1a	L1b	L1c	L2a	L2b	L3a	L3b	L3c	L4a	L4b	L4c
per sample										
89	99			99						
1										
						2				
						3				
10	1		7	1	1	95		2		7
		44	43		34		13	33	12	49
								11	16	
			14		10			5	1	
		1						1	3	
		6	9		16		6	11	2	8
		6	2		6			7	14	11
		5	3		8		11	1	1	
		3	1		3				2	1
		1	2		3		63		5	4
		2	2		3		1		1	1
		1	1					1	1	1
								1	6	
		18						5	12	12
		11	6		7		5	9		1
								2	4	
			2		1		1		2	
			7		6					
									10	
										1
										1
		2	1					6		1
								1		
									1	
								2	2	
								2		
									3	
					1					
					1				1	
										1
									1	
										1

given in parentheses along with the GenBank accession number for that most similar sequence. The percentage of clones studied from nine subjects with bacterial vaginosis (BV) and eight controls without BV. Multiple vaginal-fluid samples were obnate serial samples from the same patient. In the longitudinal study, rows above the sample designation indicate the interval have bacterial vaginosis (BV–). Thirty-five species of bacteria were detected in BV+ subjects and 12 species were detected

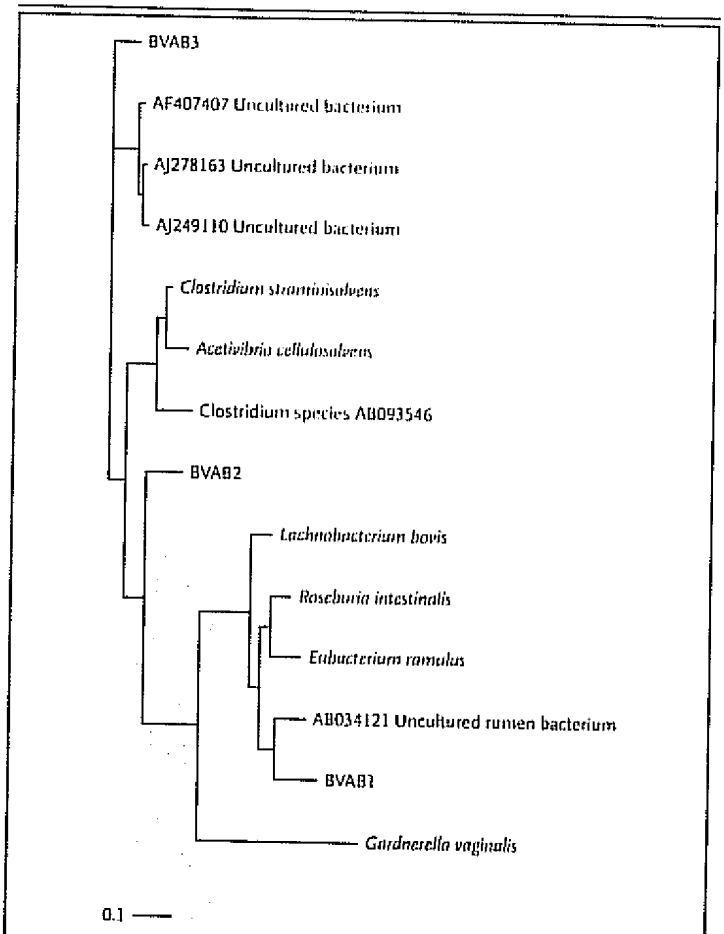

Figure 1. Phylogenetic Tree of Evolutionary Relationships among Bacteria Inferred on the Basis of Aligned 16S rDNA Sequences with the Use of a Maximum-Likelihood Algorithm.

Bacterial vaginosis–associated bacterium 1 (BVAB1), BVAB2, and BVAB3 are related to bacteria in the Clostridium phylum, though they are not closely related to any bacterium with known 16S rDNA sequence. Horizontal distances indicate evolutionary relatedness, and the bar represents 0.1 base changes per nucleotide position. GenBank accession numbers are provided for uncultured bacteria.

ed bacteria included members of the TM7 division of uncultivated bacteria and bacteria in the peptoniphilus, peptostreptococcus, gemella, aerococcus, anaerococcus, and veillonella genera.

Three newly recognized bacteria were detected only in libraries of clones from subjects with bacterial vaginosis, and we have provisionally named these organisms bacterial vaginosis–associated bacteria (BVAB) 1, 2, and 3. Identical 16S rDNA sequences from these bacteria were detected in numerous bacterial vaginosis sample libraries. A phylogenetic tree depicting the evolutionary relationships among these bacteria and their closest relatives, inferred on the basis of aligned 16S rDNA sequences, is shown in Figure 1. BVAB1, BVAB2, and BVAB3 are related to bacteria in the Clostridium phylum but are not closely related to any bacteria with known 16S rDNA sequences. For instance, 16S rDNA from BVAB1 is only 90.6 percent similar to the closest sequence in GenBank (GenBank number AB034121), which is derived from an uncultivated bacterium detected in bovine rumen.

Figures 2, 3, and 4 are a series of rank-abundance plots identifying the bacterial 16S rDNA sequence types detected in vaginal fluid and their frequency in six clone libraries from Table 1. Results from sequential, prospectively obtained samples are displayed for two subjects. In one subject, lactobacilli predominated at baseline, when bacterial vaginosis was not present, but they were replaced by a diverse community of bacteria when bacterial vaginosis was detected two months later. In the second subject, who had bacterial vaginosis at baseline, the diverse community of vaginal bacteria shifted to a predominance of lactobacilli one month after successful treatment of bacterial vaginosis.

Table 2 shows the results of bacterium-specific PCR assays of 73 samples of vaginal fluid from subjects with or without bacterial vaginosis at baseline. BVAB1 was found in 41 percent of bacterial-vaginosis samples with the use of bacterium-specific PCR but was present in up to 66 percent of clones per library (Table 1). BVAB2 was found in 89 percent and BVAB3 in 41 percent of bacterial-vaginosis samples by specific PCR, but unlike BVAB1, these bacteria were not predominant in 16S rDNA clone libraries from subjects with bacterial vaginosis. BVAB1, BVAB2, and BVAB3 were highly specific indicators of bacterial vaginosis. Two subjects without bacterial vaginosis at baseline had positive PCR assays for these bacteria (BVAB1 and BVAB2 in one and BVAB2 and BVAB3 in the other), and bacterial vaginosis developed in both within a few months after these tests.

Detection of leptotrichia species was also very specific for bacterial vaginosis. Although *G. vaginalis* was present in all subjects with bacterial vaginosis according to bacterium-specific PCR, it was also found in 59 percent of subjects without bacterial vaginosis. Other bacterium-specific PCR assays showed that atopobium species, megasphaera species, and an eggerthella-like uncultured bacterium were detected in a high percentage of subjects with bacterial vaginosis, and these assays had moder-

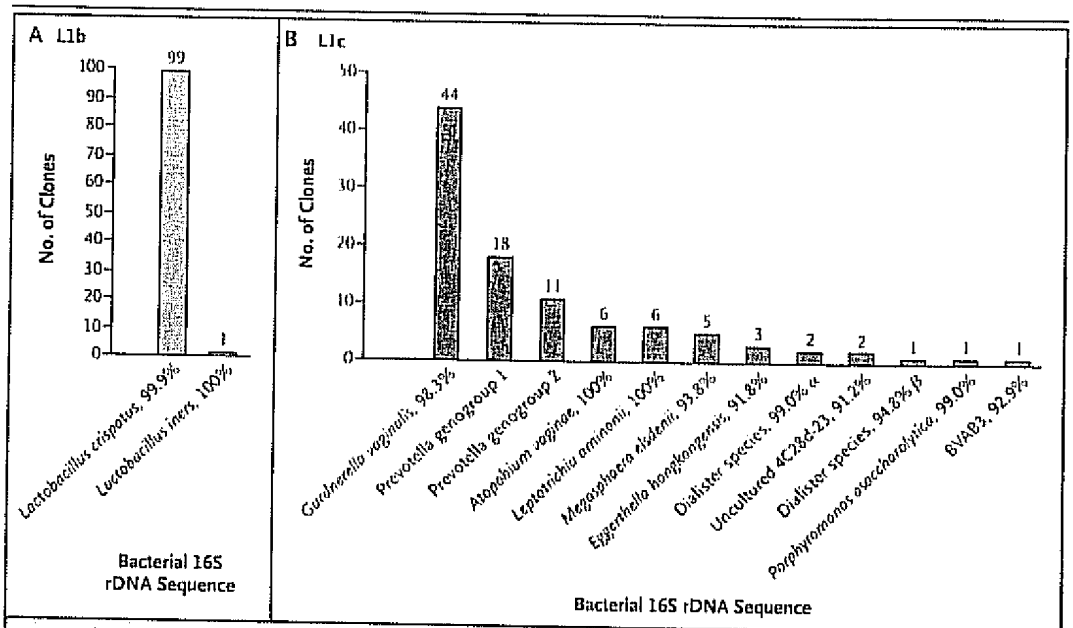

Figure 2. **Rank-Abundance Plots Showing the Percentages of Clones in the Library Corresponding to Specific Bacterial 16S rDNA Sequences in a Subject before (Panel A) and after (Panel B) the Onset of Bacterial Vaginosis.**

The labels on the x axis in each panel display the percentage of similarities between the cloned 16S rDNA sequences and the most similar 16S rDNA sequences in GenBank. Vaginal bacteria detected in one subject with the use of broad-range PCR assay of 16S rDNA are shown. *Lactobacillus crispatus* was replaced by a complex consortium of bacteria. BVAB denotes bacterial vaginosis–associated bacterium.

ate specificity for bacterial vaginosis. Detection in the vaginal fluid of each of the bacteria listed in Table 2 was significantly associated with bacterial vaginosis ($P<0.001$). Combinations of bacterium-specific PCR assays did not substantially improve sensitivity or specificity, except for the combination of BVAB1 and BVAB3, which yielded 100 percent specificity, and the combination of BVAB2 and megasphaera, which yielded 100 percent sensitivity in this small series.

FISH was performed on vaginal fluid to determine whether the bacteria detected with the use of PCR were visible by fluorescence microscopy. Table 1 of the Supplementary Appendix shows the probes used, and Figure 5 shows fluorescence micrographs of vaginal fluid from subjects with bacterial vaginosis. Thin, curved bacteria hybridizing with the BVAB1 probe were found at high density in vaginal fluid from subjects with bacterial vaginosis whose 16S rDNA clone libraries had significant BVAB1 representation. The BVAB1 probe did not hybridize with cultivated relatives of BVAB1 in the Clostridium phylum, including *Lachnobacterium bovis* and *Eubacterium ramulus*, demonstrating its specificity. In contrast, under fluorescence microscopy, BVAB2 appeared to be a short, straight rod that was wider than BVAB1, whereas BVAB3 was a relatively long, wide, straight, lancet-shaped rod. Thus, BVAB1, BAVB2, and BVAB3 have distinct morphologic features; are easily distinguished from other bacteria associated with bacterial vaginosis, such as atopobium, mobiluncus, and gardnerella species; and are found attached to vaginal epithelial cells in a way that is typical of the clue cells that characterize bacterial vaginosis.

DISCUSSION

In this study, molecular analysis of the vaginal bacterial flora showed considerable bacterial diversity in subjects who had bacterial vaginosis, with 35 bacterial phylotypes detected in the 16 baseline and longitudinal samples from subjects with bacterial vaginosis. Sixteen bacterial species detected in subjects with bacterial vaginosis seem to be newly recognized on the basis of poor homology with known 16S rDNA sequences in GenBank. Numerous bacterial genera identified in this study have not, to our

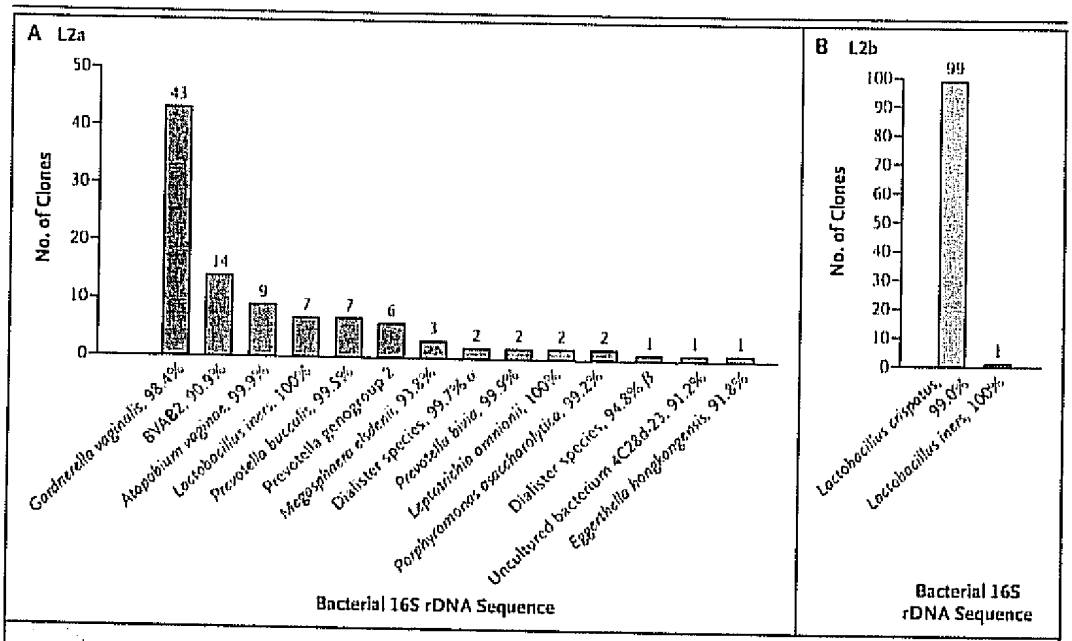

Figure 3. Rank-Abundance Plots Showing the Numbers of Clones in the Library Corresponding to Specific Bacterial 16S rDNA Sequences in a Subject before (Panel A) and after (Panel B) Successful Treatment with Antibiotics for Bacterial Vaginosis.

Vaginal bacteria are shown. Disease resolution was associated with the loss of the diverse bacterial community and colonization with lactobacillus species.

knowledge, been previously detected in the vaginal milieu with the use of cultivation methods. We found no single bacterial community in all subjects with bacterial vaginosis, but common consortia of bacteria were evident. In contrast, subjects without bacterial vaginosis had relatively homogeneous vaginal flora, and bacterial 16S rDNA sequences closely matched known cultivated bacteria in the lactobacillus genus.

The cause of bacterial vaginosis remains enigmatic. No single bacterium has been shown to cause the disease, and many cultivated bacteria linked to bacterial vaginosis, such as *G. vaginalis*, frequently colonize the vagina of women without bacterial vaginosis.[22] Our study identified several new bacterial species associated with bacterial vaginosis, but such associations do not prove causation. Additional molecular evidence may be required to assess causal associations with uncultivated bacteria.[23] Furthermore, the scenario of disease causation by a microbial community defies the traditional application of Koch's postulates. Bacterial vaginosis may be an example of polymicrobial disease causation due to the metabolic interdependence of several bacterial species in the vaginal niche.

The presence of BVAB1, BVAB2, or BVAB3 was highly specific for bacterial vaginosis in our subjects, and BVAB2 was found in 89 percent of subjects with bacterial vaginosis. We have not cultivated these microbes, and the 16S rDNA sequences from these bacteria are related only distantly to known bacteria; therefore, we have not suggested species or genus designations. Atopobium, leptotrichia, megasphaera, and eggerthella-like bacterial species were found in a high percentage of subjects who had bacterial vaginosis, according to bacterium-specific PCR. The presence of atopobium was less specific for bacterial vaginosis than was the presence of BVAB1, BVAB2, or BVAB3. Several groups have detected *A. vaginae* 16S rDNA in vaginal fluid from subjects with bacterial vaginosis and have suggested a role of this bacterium in bacterial vaginosis,[24-26] although this organism has also been detected in subjects without bacterial vaginosis.[27] As these assays are used in larger groups of women with bacterial vaginosis and those without bacterial vaginosis, their performance characteristics should be refined, and they eventually may prove useful for the microbiologic diagnosis of bacterial vaginosis.

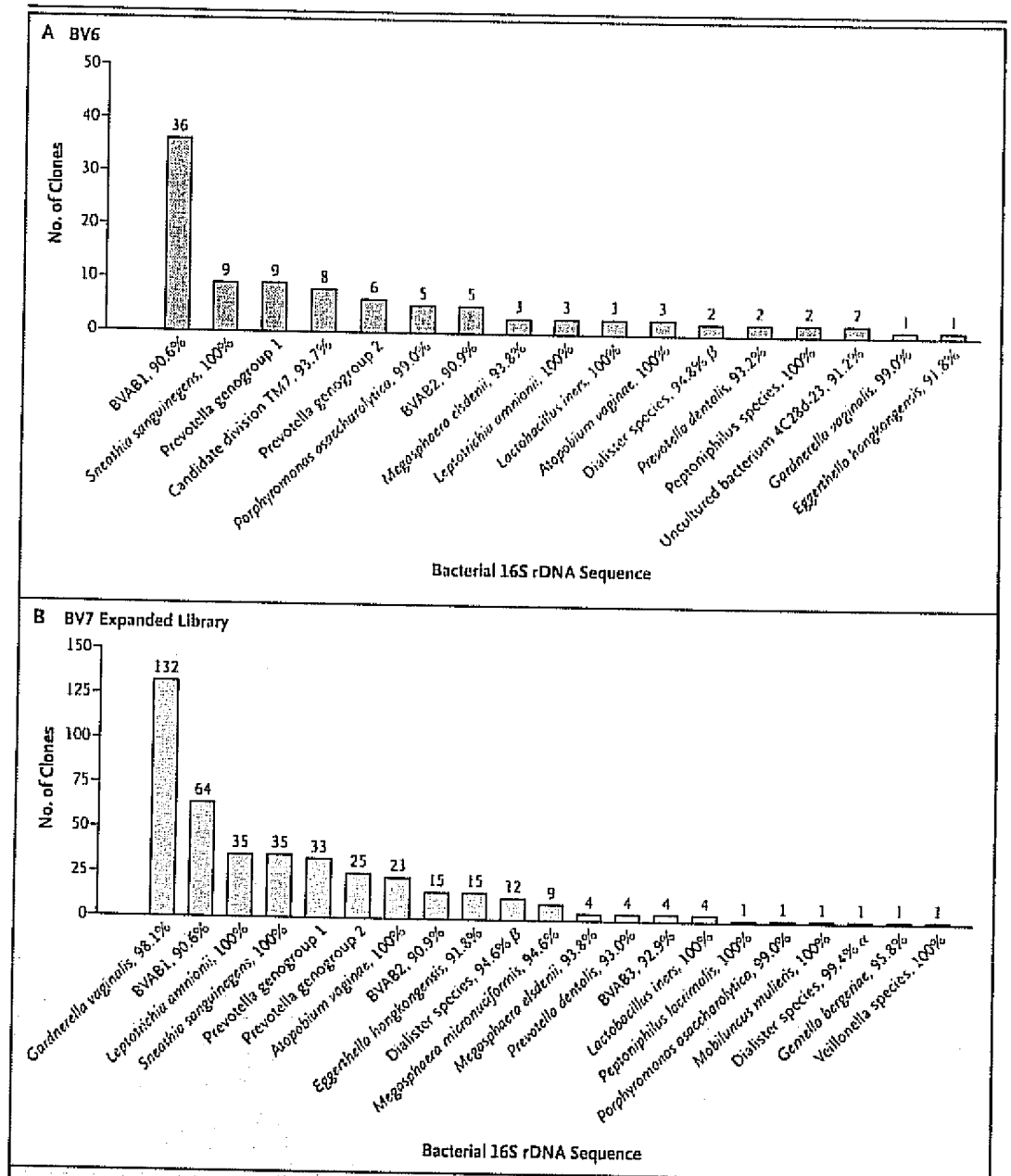

Figure 4. **Rank-Abundance Plots Showing the Numbers of Clones in the Library Corresponding to Specific Bacterial 16S rDNA Sequences.**

Single vaginal-fluid samples were studied from nine subjects with bacterial vaginosis (BV1 through BV9). Panel A shows vaginal bacteria present in a subject who had bacterial vaginosis in which BVAB1 was the dominant bacterial 16S rDNA sequence detected. Panel B shows results from a 420-clone analysis from one subject with bacterial vaginosis. BVAB1, BVAB2, and BVAB3 were present in this library, along with 18 other bacterial species.

This study has several limitations. First, broad-range 16S rDNA primers will not amplify DNA from every known and unknown bacterial species. For instance, the Bact-338F primer used in the present study does not amplify 16S rDNA from bacteria in the chlamydia genus.

Second, our findings may not be generalizable to all women with bacterial vaginosis, and the as-

Table 2. Associations between Particular Bacterial Species and Bacterial Vaginosis Based on Bacterium-Specific PCR Assay in 27 Subjects with Bacterial Vaginosis (BV) and 46 Subjects without Bacterial Vaginosis.*

Bacterium-Specific PCR Result	Bacterial Vaginosis		Sensitivity (95% CI)	Specificity (95% CI)	Unadjusted Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)
	Present (N=27)	Absent (N=46)				
BVAB1						
Yes	11	1	40.7 (22.4–61.2)	97.8 (88.5–99.9)	30.9 (3.8–1359.9)	19.0 (2.2–910.7)
No	16	45				
BVAB2						
Yes	24	2	88.9 (70.8–97.6)	95.7 (85.2–99.5)	176.0 (22.8–1862.8)	106.1 (14.3–4755.1)
No	3	44				
BVAB3						
Yes	11	1	40.7 (22.4–61.2)	97.8 (88.5–99.9)	30.9 (3.8–1359.9)	21.9 (2.5–1056.4)
No	16	45				
Gardnerella						
Yes	27	27	100.0 (89.5–100.0)	41.3 (27.0–56.8)	(5.2–∞)†	27.2 (3.9–∞)†
No	0	19				
Atopobium species						
Yes	26	9	96.3 (81.0–99.9)	80.4 (66.1–90.6)	106.9 (12.9–4493.6)	95.0 (14.5–∞)†
No	1	37				
Eggerthella-like uncultured bacteria						
Yes	25	4	92.6 (75.7–99.1)	91.3 (79.2–97.6)	131.3 (19.0–1323.6)	103.8 (13.5–4812.8)
No	2	42				
Leptotrichia species						
Yes	23	2	85.2 (70.8–97.6)	95.7 (85.2–99.5)	126.5 (18.3–1279.6)	330.6 (23.1–∞)*
No	4	44				
Megasphaera α						
Yes	26	4	96.3 (81.0–99.9)	91.3 (79.2–97.6)	273.0 (26.6–11,428.3)	134.4 (16.6–6509.8)
No	1	42				
BVAB1 and BVAB3						
Yes	9	0	33.3 (16.5–54.0)	100.0 (93.7–100.0)	(5.7–∞)†	24.7 (3.2–∞)†
No	18	46				
BVAB2 or megasphaera α						
Yes	27	4	100.0 (89.5–100.0)	91.3 (79.2–97.6)	(57.2–∞)†	190.1 (28.3–∞)†
No	0	42				

* The values represent assays of baseline vaginal-fluid samples, with odds ratios and exact 95 percent confidence intervals unadjusted and adjusted for subject age, site of clinic enrollment, presence or absence of a report of abnormal vaginal discharge, and presence or absence of a report of having had sex with men. BVAB denotes bacterial vaginosis–associated bacterium.
† The odds ratio either could not be computed or could not be computed accurately because the value approaches infinity.

sociation between the bacteria detected in this study and bacterial vaginosis should be assessed in larger numbers of women with diverse demographic characteristics and other risk factors for the disease. However, conventional methods of cultivation have yielded remarkably similar microbiologic profiles among different risk groups of women with bacterial vaginosis, including pregnant women, patients at gynecologic and STD clinics, and lesbians.[20,28-30]

Third, analysis of 100 16S rDNA clones per sample library may miss certain bacterial 16S rDNA se-

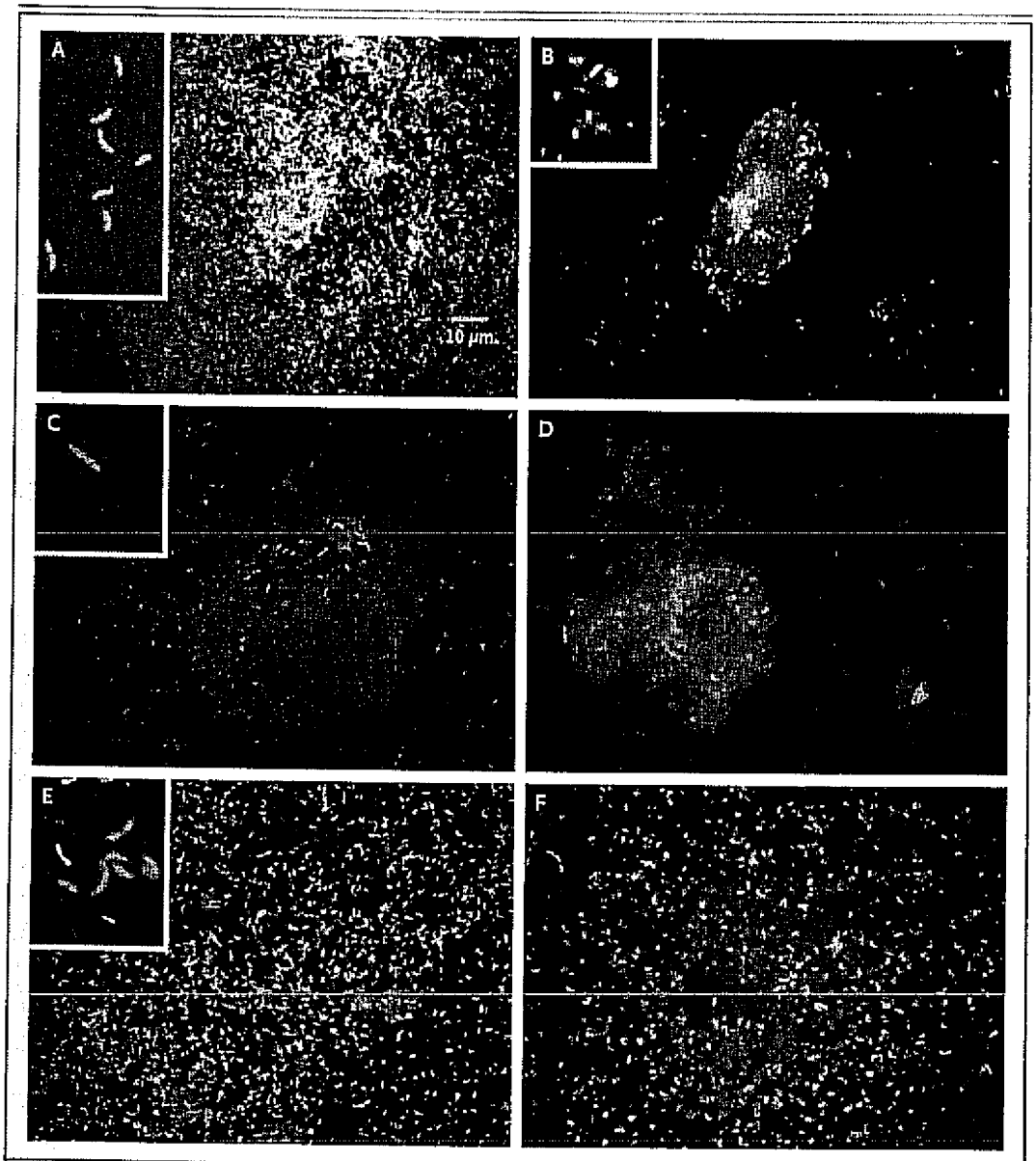

Figure 5. **Fluorescence Micrographs of Vaginal-Fluid Smears Analyzed by FISH with Labeled Oligonucleotide Probes Targeting Bacterial rRNA, with Enlargements Shown in Insets.**

Vaginal fluid from Subject 6 in the group of women with bacterial vaginosis (Panel A) shows a field of bacteria hybridizing with probes for BVAB1 (green), BVAB2 (red), and other bacteria (stained with 4′,6-diamidine-2-phenylindole, dihydrochloride [DAPI], blue). The inset in Panel A shows that BVAB1 is a thin, curved rod. A sample from the same subject shows bacteria attached to a vaginal epithelial cell (Panel B). These bacteria include organisms hybridizing with probes for BVAB1 (green) and BVAB2 (red). The cell nucleus is stained with DAPI (blue). The inset in Panel B shows that BVAB2 is a short, wide rod (red). The bacteria attached to a vaginal epithelial cell in sample L4b (Panel C) hybridize with the probe for BVAB3 (red). Other bacteria and nuclear debris stain with DAPI (blue). The inset in Panel C shows that BVAB3 is a long, lancet-shaped rod. In Panel D, a clump of bacteria from sample L4b shows many coccoid cells hybridizing with the atopobium probe (red) and other bacteria hybridizing with the broad-range bacterial probe Eub338 (blue). In Panel E, in sample L4b, bacteria hybridize with a probe for BVAB1 (green) and mobiluncus (red), but the broad-range bacterial probe Eub338 hybridizes with other bacteria (blue). The inset in Panel E shows that mobiluncus (red) is larger than BVAB1 (green) but both are small, curved rods. In Panel F, a sample from Subject 7, who had bacterial vaginosis, shows the relative proportions of BVAB1 (green), *Gardnerella vaginalis* (red), and other bacteria (DAPI, blue). *G. vaginalis* and BVAB1 were the most common clones detected in the broad-range PCR library generated from this sample.

quences present at low frequency in vaginal fluid. Although numerous statistical approaches can be used to assess species richness, we explored the effect of increasing the size of a library from 100 to 420 clones in one sample obtained from a patient with bacterial vaginosis (BV7) that was suspected to contain additional species diversity on the basis of the 5 single clones in the 100-clone library. We found that 415 of 420 clones identified (98.8 percent; see Fig. 4B) were identical to clones previously detected in the 100-clone library (Table 1, sample BV7). Five additional bacteria were present as single clones in the expanded library, increasing the total number of phylotypes detected from 16 to 21.

Fourth, different bacteria have different numbers of 16S rDNA genes per genome, and thus there is not a one-to-one relationship between the number of 16S rDNA clones detected by PCR and the number of bacteria present in a sample. Furthermore, the percentage of clones in a library should not be interpreted to indicate absolute bacterial representation, because the PCR assay is subject to amplification bias and the reaction was not stopped in the exponential phase, when it is most quantitative. Quantitative PCR and FISH methods can be used to assess bacterial representation more accurately, although one needs to know the number of rRNA operons per organism to make quantitative PCR of 16S rDNA reflect absolute bacterial counts, and these data are lacking for most of the organisms in our study.

In conclusion, bacterial vaginosis in our subjects was associated with complex vaginal bacterial communities that included many newly recognized bacterial species that have not previously been detected with conventional cultivation techniques. We identified three bacterial species in the Clostridiales order that were highly specific for the presence of bacterial vaginosis and only distantly related to known bacteria.

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EXHIBIT K

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Original article

Effect of bacterial vaginosis, *Lactobacillus* and Premarin estrogen replacement therapy on vaginal gene expression changes☆

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Abstract

The aim of the study was to investigate gene expression profiles of post-menopausal women receiving Premarin estrogen replacement therapy (ERT), compared to controls, and to examine any correlations between the bacterial vaginosis (BV) status of the subjects. Based upon an expected finding of a 50–60% difference between gene expression of host antimicrobials with alpha = 0.05 (2-sided), beta = 0.20 the calculation of 7 subjects per group, led to a sample size of 10 subjects receiving Premarin estrogen replacement therapy and 10 healthy, age-matched controls. Vaginal samples were collected at a single timepoint and processed for RNA recovery and Affymetrix array analysis, as well as Nugent scoring and denaturing gradient gel electrophoresis to identify bacteria. *Lactobacillus iners* was the most commonly detected species in the normal flora and this was confirmed with *L. iners*-specific PCR method. Vaginal swabs from 6 Premarin and 8 control vaginal samples provided a non-invasive means to analyze human gene expression. There was no significant up-regulation of cancer-associated gene expression in subject receiving Premarin ERT, but some evidence that the potentially protective innate immunity was reduced in patients with BV. Of those with a normal flora, there was a 2-fold down-regulation of carcinoma associated forkhead box A1 gene expression. BV was associated with 7-fold down-regulation of host antimicrobial colony stimulating factor, −9.83-fold for IL-1α, −8.33 for IL-1β and −3.63 for IL-6. This is the first study to use gene arrays to correlate changes in host expression response to estrogen replacement therapy and BV.

Keywords: Vaginal gene expression; Bacterial vaginosis; Lactobacillus; Premarin; Estrogen replacement therapy

1. Introduction

Oral conjugated estrogen has long been prescribed to combat the drop in hormone levels following menopause and the associated adverse side-effects, such as loss of bone density and other hormonal fluctuations. Premarin, the conjugated estrogen, is by far the most widely used hormone replacement therapy, with approximately 12 million users in 2001 within the United States alone [1]. Controversy about the benefits of estrogen replacement therapy (ERT) arose in 2002 when the Women's Health Initiative (WHI) prematurely stopped a large-scale trial of estrogen plus progestin on postmenopausal women after an increase in breast cancer and coronary heart disease incidence was noted; 26% and 29% increase respectively [2]. Since the WHI report was released, many independent studies have emerged to show estrogen therapy coupled with progestin therapy has a profoundly different effect on women than just estrogen therapy alone, reporting

☆ This study was registered as a clinical trial at http://www.clinicaltrials.gov, #NCT00318318.

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that estrogen alone has a lower incidence of breast cancer [3,4]. Despite these findings, conjugated estrogen therapy is still acknowledged as the best treatment for symptoms arising from menopausal-related decline in hormones including vasomotor symptoms, incontinence, weight gain, accelerated bone loss and changes in the vaginal mucosa [5].

One of the benefits of ERT is a restoration of the *Lactobacillus* microbiota in the vagina [6,7] and concurrent reduced incidence of urinary tract infections [8]. The loss or depletion of lactobacilli and replacement with various pathogenic organisms, such as *Gardnella vaginalis*, *Prevotella* species, *Mycoplasmas* species, *Mobiluncus* species, or *Atopobium*, results in a condition called bacterial vaginosis (BV) [9–11].

BV affects a large portion of the female population [12] and can incur various other adverse outcomes such as preterm labor and low birth rate, increased susceptibility to HIV and other sexually transmitted infections, pelvic inflammatory disease, and urinary tract infections [13–16].

The emergence of microarray genechip technology has provided an opportunity to study the impact of drugs on host tissues. Through extensive statistical data analysis software, it is possible to track changes in the expression of any gene across experimental conditions, or take a more genome-wide approach to search for any emerging patterns between genes in a given organism or site [17].

The aim of the current study was to investigate gene expression profiles of post-menopausal women receiving Premarin ERT compared to women not taking any such therapies, and to examine any correlations between the BV status of the subjects.

2. Materials and methods

2.1. Sample collection

Vaginal epithelial cell samples were collected from 20 women; 10 on oral Premarin ERT for at least two months, 10 not on any HRT, at the Victoria Family Medical Clinic in London, Ontario. The protocol was approved by the Ethics Review Board at the University of Western Ontario. Swabs were placed in 15 mL tube containing 3 mL of PAXgene stabilizer media, and suspended in solution by vortexing for 4 s. Samples were incubated for 24 h at room temperature before RNA extraction with MasterPure™ Kit (Epicentre Cat # MCR85102). The samples were divided into 1 mL aliquots centrifuged for 10 min at 13,500 × *g* and the supernatant was removed. The pellet containing the sample was then resuspended in 1 mL of DEPC water, centrifuged again for 10 min at 13,500 × *g*, and the supernatant was poured off. The cell population was over 90% epithelial cell in content, based upon microscopy.

2.2. Cell lysis and protein precipitation

The cells were lysed by adding 4 μL of 50 μg/μL Proteinase K and 300 μL of Tissue and Cell Lysis Solution and incubating in a water bath at 60 °C for 50 min, vortexing every 5 min. The samples were then placed on ice for 5 min. To precipitate out the proteins 150 μL of MPC Protein Precipitation Reagent was added and vortexed for 10 s, then separated out by vortexing for 10 min at 10,000 × *g* kept at 4 °C.

2.3. Total nucleic acid precipitation and RNA isolation

The supernatant was transferred to a clean microfuge tube and 500 μL of isopropanol was added and mixed by inverting 40 times. The total nucleic acids were pelleted by vortexing for 15 min at 10,000 × *g* kept at 4 °C and the isopropanol was carefully poured off and allowed to evaporate. Next, to remove the DNA from the sample 5 μL of DNaseI and 195 μL of 1× DNase buffer was added to the sample. The pellet was resuspended and allowed to incubate at 37 °C for 30 min. Then 200 μL of 2× T and C Lysis Solution and 200 μL of MPC Protein Precipitation Reagent were added and vortexed for 10 s then placed on ice for 5 min. Debris was pelleted by centrifuging tubes for 10 min at 10,000 × *g*. The supernatant was kept by transferring to a clean microfuge tube and 500 μL of isopropanol was added to precipitate the remaining RNA and mixed by inverting 40 times. The purified total RNA was pelleted by centrifuging at 10,000 × *g* for 15 min at 4 °C. The isopropanol was carefully poured off and allowed to evaporate. The pellet was then washed with 75% ethanol and allowed to dry. Finally, the total RNA pellet was resuspended in 20 μL of DEPC water and 35 μL of TE buffer by vortexing. RNA purity and concentration was verified by measuring the absorbance of RNA samples diluted in DEPC water at 230, 260 and 280 nm. Any samples yielding less than 100 ng of RNA were excluded. All samples were kept frozen at −70 °C until testing.

2.4. RNA quality assessment, probe preparation and GeneChip hybridization

All GeneChips were processed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA).

Biotinylated complimentary RNA (cRNA) was prepared from 100 ng of total RNA as per the Affymetrix GeneChip Technical Analysis Manual (Affymetrix, Santa Clara, CA) using the two-cycle amplification protocol. Double-stranded cDNA was synthesized using SuperScriptII (Invitrogen, Carlsbad, CA) and oligo$(dT)_{24}$ primers. Biotin-labeled cRNA was prepared by cDNA in vitro transcription using the BioArray High-Yield RNA Transcript Labeling kit (Enzo Biochem, New York) incorporating biotinylated UTP and CTP. Then 15 μg of labeled cRNA was hybridized to HGU133 Plus 2.0 GeneChips for 16 h at 45 °C as described in the Affymetrix Technical Analysis Manual (Affymetrix, Santa Clara, CA). GeneChips were stained with Streptavidin-Phycoerythrin, followed by an antibody solution and a second Streptavidin-Phycoerythrin solution, with all liquid handling performed by a GeneChip Fluidics Station 450.

2.5. GeneChip scanning and data analysis

GeneChips were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA). Signal intensities for genes were generated using GCOS1.4 (Affymetrix Inc., Santa Clara, CA) using default values for the Statistical Expression algorithm parameters and a Target Signal of 150 for all probe sets and a Normalization Value of 1.

Probe level data was imported into GeneSpring GX 7.3.1 (Agilent Technologies, Palo Alto, CA) using the RMA preprocessor.

2.6. Nugent tests

An 8 μL drop of sterile water was placed on a glass slide and a vaginal swab sample was rolled across gently and left to air dry. The slide was then Gram stained and viewed under oil immersion with a microscope. The bacterial morphotypes were then assessed and scored using a previously described Nugent scoring system; A score of 0–3 was interpreted as consistent with a Normal Gram positive rod dominated microbiota, a score of 4–6 as Intermediate, and a score of 7–10 was considered consistent with BV-like conditions in which the samples were dominated by small Gram negative and Gram-variable straight and curved rods [18].

2.7. PCR-DGGE analysis

DNA was extracted from vaginal swabs by InstaGene™ Matrix (Bio-Rad Labs, Hercules, CA), amplified by PCR using both lactobacilli-specific primers and universal eubacterial primers, and denaturing gradient gel electrophoresis (DGGE) was performed followed by band sequencing, as described previously [19].

2.8. Species-specific primers for Lactobacillus iners and Gardnerella vaginalis

Recently developed primers specific for *L. iners* by Alqumber et al. 2007 [20] were used. The PCR primer pair linersFW (5′-GTC TGC CTT GAA GAT CGG-3′) and linersREV (5′-ACA GTT GAT AGG CAT CAT C-3′) were used. The chosen primers are based upon positions 42–101 and 162–221 of the sequence AY283264. For *G. vaginalis*, these specific primers, F-GVI, 5′-TTACTGGTGTATCACTGTAAGG-3′, and R-GV3, 5′-CCGTCACAGGCTGAACAGT-3′ were used [21]. All the primers were synthesized by Invitrogen-Illumina (Invitrogen, Canada). The PCR conditions followed the same procedure as previously described [19].

2.9. Real-time quantitative RT-PCR using GAPDH as a 'house keeping' internal control gene

In order to confirm some of the findings, a select group of genes were investigated for quantitative changes in their expression. The total RNA isolated from the vaginal swabs was removed from −80 °C and the real-time qRT-PCR performed in two steps with reverse transcription converting the RNA to cDNA, using random hexamers followed by specific real-time PCR.

2.10. Reverse transcription

Reverse transcription was carried out with the superscript first-strand system. Briefly, in each tube (0.2 ml PCR tube) the total RNA (5 μg) from the samples and primer mixture comprising of 3 μl of random primer oligonucleotides (hexamers) (50 ng/μl), and 10 mM dNTP (1 μl) was added and the mixture was made up to 10 μl with DEPC H_2O. The samples were incubated at 65 °C for 5 min and then on ice for 3 min. Thereafter, the reaction master mixture comprising of 10 × RT buffer (2 μl), 25 mM $MgCl_2$ (4 μl), 0.1 M DTT (2 μl) and RNAaseOUT (1 μl) was prepared for each sample. The RNA/primer mixture was added to the reaction master mixture mixed briefly and placed at room temperature for 2 min. This was followed by addition of 1 μl (50 units) of SuperScript II RT (Invitrogen, Canada) to each sample and subsequently incubated in a temperature master cycler at 25 °C for 10 min, 42 °C for 50 min, 70 °C for 15 min and then chilled at 4 °C. The first strand cDNA was stored at −20 °C for subsequent real-time PCR.

2.11. Quantitative real-time PCR

The relative gene expression level was determined first by normalization to Human GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), which is one of the most commonly used housekeeping genes. The GAPDH primers (LUX™) (Invitrogen, life technologies, Canada) are a set of sensitive primers for performing real-time (quantitative) PCR. The primer pair includes a fluoregenic (FAM-labelled) primer and a corresponding unlabelled primer, which designed to amplify and detect a specific gene of interest (Table 1).

Before the RT-PCR, each gene of interest was initially optimized by conventional PCR with the gene specific primers. The samples with PCR product was then chosen to generate a standard curve for each gene.

2.12. RT-PCR set up

The iQTM SYBR Green Supermix (Bio-rad) was used involving 10 μl per reaction, 100 nM of each of the primers, 1.0 μl of cDNA and sterile water (DEPC H_2O) to a total volume of 20 μl per tube. The standard curves were generated by serial dilutions (1:10) using the PCR products for the GAPDH and the specific genes. The RT-PCR followed a program in Rotor-Gene 6000 (Corbett Research, Australia) for initial denaturation at 95 °C for 5 min and cycling at 95 °C for 10 s, annealing at 60 °C for 15 s and extension for 20 s. the cycle was repeated 35 times. Finally the products were melted with temperature from 72 °C to 95 °C rising by 1 °C each step. The relative value of the gene expression was quantified with the Rotor-Gene 6000 soft-ware version 1.7.75.

Table 1
Primer pairs used for the RT-PCR for each gene of interest

Human cDNA target		Primer sequence	Ref
Secretoglobulin	Forward	CTGAGCTCACAGCAAAAC	[22]
	Reverse	GAGCTGGGCAGAAC	
IL-1beta	Forward	TACGAATCTCCGACCACCACTACAG	[23]
	Reverse	TGGAGGTGGAGAGCTTTCAGTTCATATG	
Lactotransferrin	Forward	CTCCAGACCGCAGACATGAA	[24]
	Reverse	CTGGGAGGAGAAGGCACATT	
BCAS-1	Forward	AGAAGGACTGGAGACTGCAAAG	[25,26]
	Reverse	TAAGGTCAGCTGAAGTGGTGG	

3. Results

3.1. Bacteria-vaginal samples

Lactobacillus iners was the predominant *Lactobacillus* found in over 50% samples (Table 2), and all samples receiving hormone therapy had one or more species of *Lactobacillus*, whereas half of the control samples contained no detectable amounts of these species (data not shown). The predominance of *Lactobacillus iners* was confirmed with species-specific primers. Among the Premarin group 6 out of 10 (60%) had *L. iners*, while in the control group 5 out of 10 (50%) had *L. iners*. From the ten Premarin samples seven had a normal Nugent score of 0–3, three had an intermediate score of 4–6, and none had BV score of 7–10. In contrast, the control samples not receiving hormone therapy had four normal scores, one intermediate, and five with BV. The difference in the distribution of the Nugent scores (Normal:Intermediate:BV) between controls and Premarin-treated subjects was statistically significant ($P = 0.033$) (using Pearson Chi-Square, SPSS 14 for Windows, SPSS Inc. Chicago, IL). Among the 5 controls who had BV, 4 (80%) were negative for *L. iners*, while only 4 of out 10 (40%) in the Premarin group were negative for *L. iners*. The DGGE testing did not uncover the most prominent anaerobic organisms associated with BV, and this may have been due to the selection of eubacteria primers used. However, using PCR species-specific primers led to retrieval of more *Gardnerella vaginalis* in the control group (4/10) compared to 1/10 with eubacterial PCR-DGGE. Similarly, *Gardnerella vaginalis* was recovered in one of the patients with intermediate BV in the Premarin group, which DGGE failed to identify.

Table 2
Summary of Nugent scores and bacterial species recovered from vaginal samples

Controls	Nugent	Lactobacilli	Presence of *L. iners* with specific primers	Eubacteria	*Gardnerella vaginalis* specific primers
8001	BV		Negative	*E. coli*	Negative
8002	Normal	*L. iners*	Positive		Negative
8004	BV		Negative	*S. agalactiae*	Negative
8005	Normal	*L. crispatus*	Positive		Positive
8009	BV	*L. iners; L. jensenii*	Positive	*G. vaginalis; S. agalactiae*	Positive
8011	BV		Negative	*S. agalactiae*	Negative
8014	Intermediate		Negative	*S. agalactiae*	Negative
8016	Normal	*L. iners; L. crispatus*	Positive		Positive
8017	Normal	*L. iners*	Positive		Positive
8019	BV		Negative	*S. agalactiae*	Negative
Premarin					
8003	Intermediate		Negative	*S. agalactiae*	Positive
8006	Normal	*L. iners; Lactobacillus* sp.	Positive		Negative
8007	Normal	*L. iners*	Positive		Negative
8008	Normal	*L. gasseri; L. iners*	Positive		Negative
8010	Intermediate	*L. jensenii*	Negative	*S. agalactiae*	Negative
8012	Intermediate	*L. iners; L. jensenii*	Positive	*S. agalactiae*	Negative
8013	Normal	*L. iners; L. crispatus*	Positive		Negative
8015	Normal	*L. crispatus*	Negative		Negative
8018	Normal	*L. iners*	Positive		Negative
8020	Normal	*L. crispatus*	Negative		Negative

3.2. Array studies

From the vaginal swabs collected from the 20 subjects, six were excluded from GeneChip analysis due to low concentration of total RNA below 100 ng/μL: two samples from the control group and four from the Premarin group. The average age of the subjects was 55.6 years (54.8 in controls and 56.3 in Premarin group).

Once data entry, normalization, and transformation were complete, GeneSpring GX 7.3.1 displayed the 54, 675 different probesets corresponding to 54,613 transcripts of relative expression in the Premarin group with respect to the mRNA expression in the control group. Initially a volcano plot was performed for the entire genome, filtering out genes by a threshold expression value as well as a threshold P-value of significance. In this case, values were set to filter genes that had a differential gene expression in the Premarin group by ±1.5-fold change, and a T-test P-value of 0.05 or less. This resulted in a gene list of 227 genes, all possessing a significantly different mRNA expression in the Premarin group (supplemented material).

Next the 227 genes were organized by known function using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Functions included anti-apoptosis, apoptosis, antimicrobial response, bacterial defense response, cell cycle arrest, innate immune response, chemokines, interleukins,

and oncogenes. The KEGG gene lists made by functions relating to anti-apoptosis, apoptosis, bacterial defense response, cell cycle arrest, innate immune response, chemokines, displayed few or no genes with differential expressions that passed the filter. However, genes relating to antimicrobial response, interleukins, and oncogenes did show differences that warranted further analysis.

3.3. Oncogenes

Using the KEGG annotation for genes relating to cancer, a gene list was made of significantly different expressed genes in the Premarin group, all of which were down-regulated compared to controls (Table 3). To assess whether the higher BV rates in the control group skewed the results, a second cancer gene comparison was made only using samples which had normal Nugent scores between the control and Premarin groups. Again, mRNA down-regulation was observed, albeit to a lesser extent. In a third comparison, samples within the control were tested based on their Nugent scores. This analysis showed that the same genes were all slightly up-regulated in subjects with BV.

Various genes found reported to be associated with different types of breast carcinoma [27,28], namely CHEK2 checkpoint homolog, tumour protein p53, breast cancer 1 and 2 (BRCA1,2), ataxia telangiectasia (ATM), RAD51 homolog, V-erb-b2 erythroblastic leukemia viral oncogene homolog2 (ERBB2), insulin-like growth factor (IGF-1), telomerase reverse transcriptase (TERT) and prolactin (PRL) showed no significant differences, but forkhead box A1 (FOXA1) was down-regulated 2-fold. To account for the higher prevalence of BV in the control group, a comparison of BV versus normal Nugent scores, irrespective of grouping, showed no differences, nor did an analysis of normal versus BV in the control group, except for FOXA1 (3.79 fold up-regulated).

3.4. Antimicrobial response

Among the antimicrobial response genes differentially expressed significantly by ±1.5-fold and P-value <0.05, three genes known for antimicrobial properties had relevant immune function: colony stimulating factor (CSF), lactotransferrin, and secretoglobin. These genes were up-regulated in subjects receiving oral Premarin (Table 4). A sub-analysis comparing BV versus normal Nugent scores showed a 7.07 fold down-regulation of the CSF3 receptor and no significant up-regulation of lactotransferrin or secretoglobin (Table 5).

3.5. Interleukins

The analysis of interleukin gene expression showed no consistent pattern, with less than 2-fold up-regulation of IL-1 and IL-6 in Premarin treated subjects compared to controls (Table 6). Comparing normal samples in control and Premarin groups (eliminating BV as a variable), resulted in down-regulation of IL-1α and IL-1β by 1.61 and 1.94 fold respectively. This down-regulatory trend was more apparent comparing controls with BV subjects, and there was significant down-regulation of IL-1α (9.83 fold), IL-1β (8.33), and IL-6 (3.63).

3.6. RT-PCR

Using RT-PCR, and normalized with the human GAPDH as internal control (Fig. 1), the results obtained from the microarray data have been confirmed, for example secretoglobulin was up-regulated in the Premarin group compared with the control group (100.17 vs 68.32). Also lactotransferrin was up-regulated by 5-fold in the Premarin group (30.66 vs 6.10). IL-1beta was confirmed to be down-regulated in BV patients, as shown in Table 7.

Table 3
Using the KEGG annotation for genes relating to cancer, a gene list was made of significantly different expressed genes in the Premarin versus control groups, and comparing Nugent normal and BV samples, is shown

Common gene symbol	Description	Fold change for expression of annotated oncogenes between control ($n = 6$) and Premarin ($n = 6$)	Fold change when comparing only the normal Nugent scores between control ($n = 3$) and Premarin ($n = 4$)	Fold change comparing normal ($n = 3$) and BV ($n = 4$) samples within the control group
BNIPL	BCL2/adenovirus E1B 19 kD interacting protein like	−4.08	−1.62	2.65
TNS3	Tensin 3	−2.48	−1.34	2.70
SMAD2	SMAD, mothers against DPP homolog 2 (Drosophila)	−2.48	−1.68	1.57
HCAP-G	Chromosome condensation protein G	−2.33	−1.82	1.41
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	−2.31	−1.72	1.54
FRK	fyn-related kinase	−3.17	−2.54	1.84
BARD1	BRCA1 associated RING domain 1	−2.28	No change	No change
BCAS1	Breast carcinoma amplified sequence 1	−3.98	−1.89	2.39
BCAS2	Breast carcinoma amplified sequence 2	−2.63	1.05	1.28

Table 4
Difference in mRNA expression of genes relating to antimicrobial properties when comparing the Premarin ($n = 6$) to control ($n = 8$) group

Gene symbol	Fold change	Description
CSF3R	2.43	Colony stimulating factor 3 receptor (granulocyte)
CSF3	1.68	Colony stimulating factor 3 (granulocyte)
LTF	7.76	Lactotransferrin
SCGB1D2	5.82	Secretoglobin, family 1D, member 2
SCGB2A2	13.08	Secretoglobin, family 2A, member 2

Relevant genes show up-regulation for subjects on hormone therapy. (P-value <0.05).

4. Discussion

This is the first study to examine gene expression changes in the vagina of women receiving ERT. It is also the first to examine vaginal cell gene expression changes between subjects with BV compared to a normal lactobacilli dominated microbiota. The high recovery of *Lactobacillus iners* is confirmatory with previous studies [7,19]. Although the sample size is too small to state incidence and prevalence rates, it was apparent that BV was much more common amongst non-ERT subjects. The study equally highlighted the importance of using PCR reactions with species-specific primers in the recovery of associated organisms. It also showed that *L. iners* was not associated with BV, as others have suggested [29,30].

Of special interest were any changes in cancer-related genes and inflammation genes, as these can be influenced by estrogen [31–34]. Among the filtered genes relating to cancer that were expressed differently in the two studied groups, two genes warrant particular discussion. The first is breast carcinoma amplified sequence 1 (BCAS1). This stretch of roughly 1 megabases is thought to contain 5 genes, one of which is NABC1 which predicts to encode a 585-amino acid protein of unknown function [35]. This unknown NABC1 gene, as well as the entire BCAS1 region has been shown to be highly over-expressed in many types of breast cancers, as well as being associated with aggressive tumor behaviour. It was down-regulated 4-fold in subjects taking Premarin. The other oncogene within the filtered list is breast carcinoma amplified sequence 2 (BCAS2), an estrogen receptor α (ERα) interacting

Table 5
Relative mRNA expression of genes relating to antimicrobial function when comparing normal ($n = 3$) and BV ($n = 4$) samples within the control group

Gene symbol	Fold change	Description
CSF3R	−1.81	Colony stimulating factor 3 receptor (granulocyte)
CSF3	−7.07	Colony stimulating factor 3 (granulocyte)
LTF	1.01	Lactotransferrin
SCGB1D2	1.16	Secretoglobin, family 1D, member 2
SCGB2A2	1.89	Secretoglobin, family 2A, member 2

Table 6
Expression changes in proinflammatory interleukins between control ($n = 8$) and Premarin ($n = 6$) samples

Gene symbol	Fold change	Description
IL1B	1.95	Interleukin 1, beta
IL6	1.53	Interleukin 6 (interferon, beta 2)
IL6R	1.85	Interleukin 6 receptor
IL1A	1.69	Interleukin 1, alpha
IL7	−1.60	Interleukin 7
IL20RA	−1.63	Interleukin 20, receptor alpha
IL19	−1.66	Interleukin 19

protein [36]. BCAS2 is also found to be over-expressed in many types of breast carcinomas and may be important to tumor development by increasing the estrogen receptor function. Again, within the group taking oral Premarin, there was a down-regulation of mRNA for this gene by 2.6-fold. Likewise, BCAS-1 was down-regulated 4 fold, a result more than confirmed by TR PCR. The extent to which these findings have clinical ramifications remains to be seen.

Findings from the meta-analysis of gene expression associated with the risk of breast carcinoma produced a list of 12 genes of interest. However, ERT had little or no effect on their expression in the vaginal cells studied here. The exception was forkhead-box A1 (FOXA1), a transcriptional factor which has recently been attributed to activating tumor suppressor gene p27, and thus plays a critical role in the cellular progression to oncogenesis [37,38]. The up-regulation of this gene in subjects with BV is of interest, and potential mechanisms are worthy of further investigation.

Lactotransferrin, a member of the iron-binding transferrin proteins known to have antimicrobial properties, was first identified in breast milk but is now known to play an important role in the mucosal immune response [39]. Studies show breast milk enriched with lactotransferrin inhibits the growth of *Escherichia coli* [40]. Lactotransferrin functions as an antibacterial protein by aiding with neutrophil function by means of regulating hydroxyl radical production as well as being involved in the secretion of IgA antibodies. The up-regulation

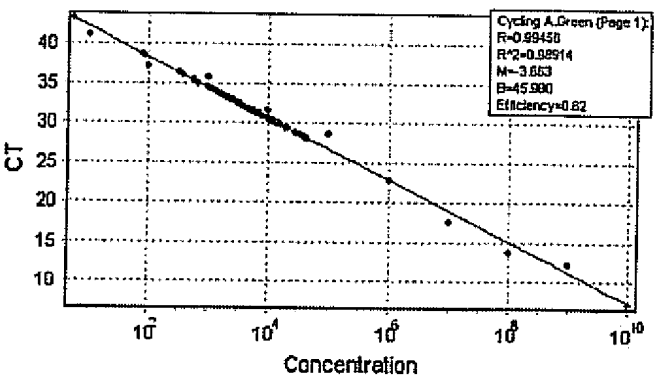

Fig. 1. Standard curve for GAPDH with cDNA of the samples. *Blue square dots represents GAPDH standard, while the red square dots shows cDNA sample data points.

Table 7
Relative quantitative expression of specific genes in the vagina of women treated with Premarin and normalized with GAPDH as a 'housekeeping' internal control gene

Control group		Secretoglobulin	Lactotransferrin	IL-1beta	BCAS-1
8001	BV	36.33	0.54	0.04	0.50
8002		4.25	0.18	0.01	0.20
8004	BV	0.47	0.23	0.01	0.16
8005		8.46	2.05	0.37	1.02
8009	BV	2.63	0.82	0.09	0.54
8011	BV	6.67	0.55	0.01	1.74
8014		3.22	0.65	0.01	2.35
8016		4.07	0.32	0.13	1.63
8017		1.68	0.53	0.06	2.71
8019	BV	0.54	0.23	0.01	0.91
Total sum		68.32	6.10	0.74	11.76
Premarin group					
8003		0.45	0.20	0.00	0.29
8006		0.52	0.14	0.23	0.11
8007		2.72	0.18	0.00	0.09
8008		0.60	0.19	0.00	0.07
8010		4.43	1.31	0.02	1.14
8012		8.53	2.05	0.03	14.52
8013		0.84	0.15	0.00	0.57
8015		2.20	0.48	0.01	1.87
8018		20.26	5.87	0.07	20.84
8020		59.62	20.09	0.23	95.25
Total sum		100.17	30.66	0.64	134.75

Results are expressed as a ratio.

of lactotransferrin mRNA by 7.8-fold in subjects receiving Premarin suggests that ERT may provide a more alert and functional antimicrobial response against infection. The RT PCR data showed that lactotransferrin was present in highest levels in five subjects with a Normal or Intermediate Nugent score, again suggestive of some anti-microbial effects.

Filtering out genes based on fold change greater than 2.0 and a *P*-value of less than 0.05, two members of the secretoglobin family, 1D and 2A, were up-regulated 5.8 and 13.0-fold respectively in the subjects receiving Premarin. Secretoglobin is a protein found in the mucosa of many vertebrates and although its specific biological activities are unknown, it has demonstrated antimicrobial properties [41]. It was first discovered in the lung, but has since been found expressed in the endometrium, prostate and seminal vesicles, and its expression is regulated by steroid hormones. The overall up-regulation found here may be further evidence of increased natural defenses due to Premarin therapy and the recovery of a *Lactobacillus*-dominant vaginal microbiota and less recovery of *Gardnerella vaginalis* with species-specific primers, an organism usually linked with BV. However, such a conclusion needs further verification, as the RT PCR data showed that the highest levels of secretoglobulin (ratio >8) were found in four Normal or Intermediate Nugent score subjects, but also in one with BV (#8001).

Colony stimulating factor (CSF), from a family of glycoproteins essential for the proliferation and activation of neutrophils [42] and linked to localizing infection and sepsis [43], and its receptor, were considerably down-regulated in subjects with BV, suggesting a hindered immune response in postmenopausal women.

Interleukins play a critical role in the innate immune response within the human body, and more specifically, the vaginal mucosa [44]. Presence of these potent cytokines elect immunological responses such neutrophil migration, inflammation, and differential gene expression ultimately leading to activation of other defense mechanisms [17,32]. IL-1, comprised of two subunits α and β, is a proinflammatory interleukin present at basal levels and is thought to play a key role in activating complimenting cascades. The present findings of an up-regulation of IL-1 of roughly 2-fold for subjects on ERT may suggest that the recovery of *Lactobacillus* helps to maintain an anti-infective response. The down-regulation of IL-1 mRNA expression in control subjects may suggest a deficiency in host defenses during periods of heightened pathogenic bacterial infection. The significant 8–9 fold down regulation of IL-1 mRNA expression in subjects with BV was somewhat surprising, but may indicate that the host was not responsive, or may have become tolerant to the BV organisms. This might explain why so many cases of BV are not associated with symptoms and signs of infection [12,45]. On the other hand, clinical data have shown an increased incidence of BV after menopause [12], which would support the concept of a weakened innate immunity after menopause, and explain why premenopausal women with BV have a significantly elevated IL-1β response [46].

In summary, vaginal swabs provided a non-invasive means to analyze the vaginal microbiota and human gene expression in post-menopausal women. There was no evidence of significant up-regulation of cancer-associated gene expression in subject receiving Premarin ERT, but some evidence that the potentially protective innate immunity was reduced in patients with BV.

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Appendix A. Supplementary material

Supplementary information for this manuscript can be downloaded at doi: 10.1016/j.micinf.2008.02.007.

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EXHIBIT L

FAO Technical Meeting Report

Food Quality and Standards Service
Food and Agriculture Organization of the United Nations

FAO Technical Meeting on PREBIOTICS

FAO TECHNICAL MEETING ON
PREBIOTICS

Food Quality and Standards Service (AGNS)
Food and Agriculture Organization of the United Nations (FAO)
September 15-16, 2007

This report was prepared for the Food Quality and Standards Service (AGNS), Food and Agriculture Organization of the United Nations (FAO) based on the technical meeting convened by AGNS/FAO (FAO secretariat: Maya Pineiro, Senior Officer, AGNS) with the international experts namely: Nils-Georg Asp, Oscar Brunser, Sandra Macfarlane (Chair), Lorenzo Morelli, Gregor Reid and Kieran Tuohy (Rapporteur).

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1. Objective of the Meeting

This **Technical Meeting** of experts was convened to begin discussions on guidelines, recommended criteria and methodology for conducting a systematic approach for the evaluation of prebiotics, leading to their safe and efficacious use in food. The purpose was to discuss the prebiotic concept and its application to human health. An aim was to determine if prebiotics is an area of food research which would benefit from an Expert Consultation drawn from independently recognised leading experts convened under the auspices of the FAO.

Prebiotics have become a recognised functional food commodity. The Technical Meeting concluded that advances in prebiotic research provide sufficient substance for the FAO to consider a full Expert Consultation.

2. Current prebiotic standing and state of the art

Currently, there are no industry-wide accepted guidelines governing the usage of the term prebiotic on food products. The market for prebiotics in food is growing rapidly. A 2007 report on the world prebiotic market states that there are over 400 prebiotic food products and more than 20 companies producing oligosaccharides and fibres used as prebiotics [1]. A Frost & Sullivan review reported that the European prebiotics market is currently worth €87 million, and will reach €179.7 million by 2010. This is a dramatic growth spurt, in part explained by the increase in diversity of food products to which prebiotics have been added.

The basis for the expanded use of prebiotics is several-fold, not the least of which is a belief that modern day humans do not ingest sufficient quantities of lactic acid bacteria or their growth stimulants including non-digestible carbohydrates. In addition, there is a growing recognition that events taking place in the intestine and influenced by microbes, have major consequences for human health. Thus, not only are prebiotics being examined for anti-pathogenic effects (such as inhibiting adhesion of pathogenic organisms to the gut mucosa), but they are also being developed to decrease faecal transit time, lower cholesterol and the glycaemic response, improve bone health, lower daily energy (fat) intake, relieve symptoms of inflammatory bowel disease, and attempt to lower colon cancer rates [2]. The latter effects are also promoted for dietary fibres, and this raises the question of if and how prebiotics are differentiated from, or the same as, dietary fibres.

A prebiotic was originally defined in 1995 as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" [3]. A more recent definition stated that "A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health" [4].

The principal concept associated with both of these definitions is that the prebiotic has a selective effect on the microbiota that results in an improvement in health of the host. The definitions arose from observations that particular dietary fibres bring about a specific modulation of the gut microbiota, particularly increased numbers of bifidobacteria and/or lactobacilli, and that ingestion of these compounds was associated with improved host health. However, as our ability to determine the microbial ecology of the gastrointestinal microbiota

increases, along with our understanding of how this complex and diverse collection of bacteria functions, we now recognise that a beneficial modulation of the microbiota encompasses far more than bifidogenesis.

Common prebiotics in use include inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), soya-oligosaccharides, xylo-oligosaccharides, pyrodextrins, isomalto-oligosaccharides and lactulose. The majority of studies have so far focused on inulin, FOS and GOS [5, 6]. These saccharides have now a long history of safe use and are generally regarded as safe, although there is some concern over increased gas production with some compounds, particularly when ingested in higher amounts or during the first few days of intake.

There is also a range of new prebiotic compounds emerging, and these include: pecticoligosaccharides, lactosucrose, the sugar alcohols, gluco-oligosaccharides, levans, resistant starch, xylosaccharides and soy-oligosaccharides. These compounds have been studied to varying degrees *in vitro*, in animal feeding studies, but rarely in human feeding studies. Novel compounds new to the human diet fall under the European regulatory category of "novel foods" and will require legislated levels of safety and toxicological assessment before they can be included in food products. However, little legislation exists governing the use of the word "prebiotic" itself on functional food products and there is a growing collection of commercially available products which bear the prebiotic label but for which supportive scientific literature is sparse or lacking all-together.

The call for a scientific evaluation of the functional and health properties of prebiotics is thus timely. The FAO Technical Meeting on Prebiotics addressed guidelines, recommended criteria and methodologies for conducting a systematic approach for the evaluation of prebiotics leading to their safe and efficacious use in food.

3. Defining the term prebiotic

The existing definitions of a prebiotic, as stated above, while differentiating this class of non-digestible food ingredient within the dietary fibres and broadly serving the more common and well studied prebiotic oligosaccharides, is restrictive in its applicability for target sites outside the gastrointestinal tract. It is also restricted by necessitating a single mechanism of action (e.g. anti-adhesive activities) in addition to the selective changes in the composition and/or activity in the gastrointestinal microbiota. These definitions too, were drawn up early in the current wave of interest surrounding the impact of the gut microbiota on human health and disease, specifically, before metagenomic demonstration of the high species richness, novelty (with up to 70% of the gut microbiota commonly categorised as "new to science" upon direct 16S rRNA gene fragment sequencing) and degree of metabolic cross-feeding or co-dependence within the gut microbiota.

The stipulation of selective fermentation or selective increase in growth and/or activity encompassed within these current definitions, has become synonymous with the preferential increased abundance of bifidobacteria and/or lactobacilli. However, this is now inadequate to describe a beneficial modulation of a microbiota dominated by members of the *Clostridium coccoides*, *C. leptum* groups and the *Bacteroides*, regarded as key species together with the bifidobacteria in saccharolytic fermentation within the colon. These considerations and their implications warrant a reconsideration of the prebiotic definition itself.

The Technical Meeting proposes a broader definition to encompass new prebiotics, and to more accurately reflect current understanding of the microbial ecology of the human microbiota. This revised definition follows.

3.1. Definition

A prebiotic is a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota.

3.2. Qualifications

1. Component – not an organism or drug; a substance that can be characterized chemically; in most cases this will be a food grade component.
2. Health benefit – measurable and not due to absorption of the component into the bloodstream or due to the component acting alone; and over-riding any adverse effects
3. Modulation – show that the sole presence of the component and the formulation in which it is being delivered changes the composition or activities of the microbiota in the target host. Mechanisms might include fermentation, receptor blockage or others.

A prebiotic can be a fibre but a fibre need not be a prebiotic.

4. How to evaluate and substantiate that a product is a prebiotic

4.1. Product specification/characteristics of the prebiotic

The component to which the claim of being prebiotic is attributed, must be characterized for any given product. This includes:

- Source, origin
- Purity
- Chemical composition and structure
- Vehicle, concentration and amount in which it is to be delivered to the host

4.2. Functionality

At a minimum, there needs to be evidence of a correlation between the measurable physiological outcomes and modulation of the microbiota at a specific site (primarily the gastrointestinal tract, but potentially also other sites such as vagina and skin). Need to correlate a specific function at a specific site with the physiological effect and its associated timeframe.

- Within a study, the target variable should change in a statistically significant way and the change should be biologically meaningful for the target group consistent with the claim to be supported.

- Substantiation of a claim should be based on studies with the final product type, tested in the target host.
- A suitably sized randomized control trial (compared to placebo or a standard control substance) is required, preferably with a second independent study.
- Examples of physiological outcomes due to administration of prebiotics could be: satiety (measured towards carbohydrates, fats, total energy intake); endocrine mechanisms regulating food intake and energy usage in the body; effects on absorption of nutrients (e.g. calcium, magnesium, trace elements, protein); reduced incidence or duration of infection; blood lipid and classic endocrine parameters; bowel movement and regularity; markers for cancer risk; changes in innate and acquired immunity that are evidence of a health benefit.

4.3. Qualifications

- Bifidogenic effects are not sufficient without demonstrated physiological health benefits.
- It is recognized that at this time, determining events that take place within compartments of the intestine are often difficult. Until such times as specific site sampling or more sophisticated methods can reliably link microbiota modulation with health benefits, faecal analysis will be deemed suitable, with limitations.

4.4. Safety

As with any food component, safety parameters are established by all national regulations. It is recommended that the following issues need to be covered in any safety assessment of a prebiotic final product formulation:

- If , according to local legislation, the product has a history of safe use in the target host, such as GRAS or its equivalents, then it is suggested that further animal and human toxicological studies may not be necessary.
- Safe consumption levels with minimal symptoms and side effects should be established.
- The product must not contain contaminants and impurities.
- Based upon current knowledge, the prebiotic should not alter the microbiota in such a way as to have long term detrimental effects on the host.

5. Management issues

- **Production** – the onus is on the manufacturer to ensure substances considered prebiotics should have purity and consistency in composition between product lots.
- **Formulation and storage** – It is recommended that the limit of stability in different product types, effects of processing and production technologies on prebiotic composition, and the desired biological activity in the target host be evaluated.
- **Regulatory** – Prebiotics are components designed for specific health effects through modulation of the host microbial population. The onus is on the producer to provide the regulatory agency where sales are to be made with an appropriate level of documentation supporting the health claims. It is possible that these may refer to

disease prevention, treatment and risk reduction claims. A number of documents available in the public domain, such as PASSCLAIM, EFSA guidelines and others [7,8], provide criteria for evaluating the quality of data suitable for making health claims on food and food components.

- The status of prebiotics is not established on an international basis. The term prebiotic must be used only when a health benefit related to modulation of the target site microbiota has been demonstrated in the target host.
- The issues of product specific testing were considered. The consensus was that the onus should be on the producer to show that a new formulation e.g. yoghurt, is equivalent to the one (e.g. dried powder) proven in target host studies to confer the prebiotic effect.

6. Monitoring

The Technical Meeting recommends that prebiotic producers, medical professionals and public health officers consider some form of system to monitor the health outcomes of long-term prebiotic administration. This is suggested as a means to gain insight into potential side effects as well as assess long-term benefits. A necessary prerequisite for surveillance is a proper trace-back system.

7. Future research areas

- It is recognised that there are numerous potential new applications being considered for prebiotic use e.g. prevention and or management of type 2 diabetes mellitus; drug bioavailability; effects on autoimmune diseases and allergy; modulation of pathogenic biofilms. There is a need for more randomised, placebo controlled clinical trials with adequate statistical power. We encourage publication in peer-reviewed journals of all clinical trials, whether the outcome is positive, negative or adverse.

- It is recognised that prebiotics may be used in conjunction with probiotics; this is considered a synbiotic. Depending on the nature of the two components, the net effect may not be synergistic. We recommend that the term synbiotic only be used if the net health effect is synergistic. It is also recommended that the issue of synbiotics be addressed by a separate Technical Meeting.

Figure 1:

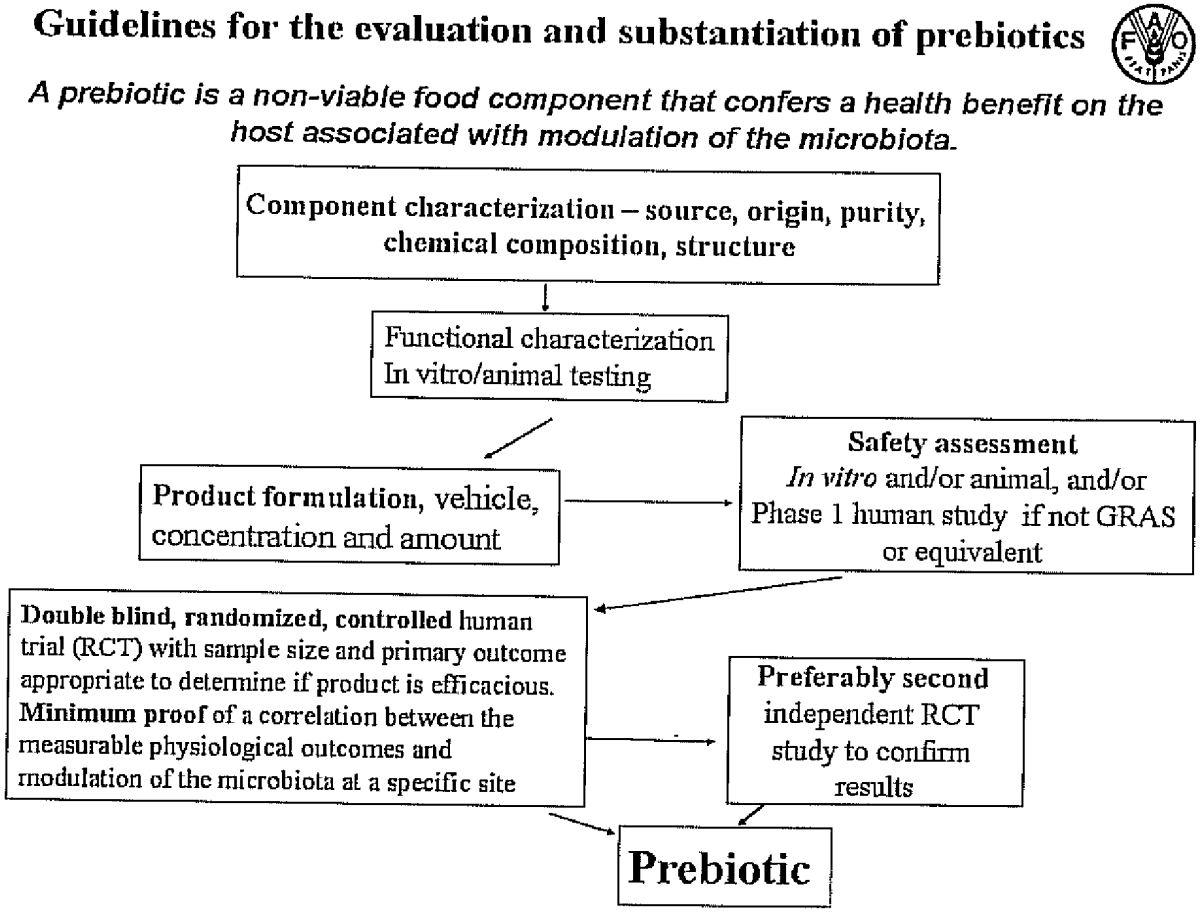
Guidelines for the evaluation and substantiation of prebiotics
A prebiotic is a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota.
Component characterization – source, origin, purity, chemical composition, structure
Functional characterization In vitro/animal testing
Product formulation, vehicle, concentration and amount
Safety assessment In vitro and/or animal, and/or Phase 1 human study if not GRAS or equivalent
Double blind, randomized, controlled human trial (RCT) with sample size and primary outcome appropriate to determine if product is efficacious. Minimum proof of a correlation between the measurable physiological outcomes and modulation of the microbiota at a specific site
Preferably second independent RCT study to confirm results
Prebiotic

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EXHIBIT M

Prevalence of Bacterial Vaginosis

2001–2004 National Health and Nutrition Examination Survey Data

Jenifer E. Allsworth, PhD, and Jeffrey F. Peipert, MD, MPH

OBJECTIVE: To estimate the prevalence and correlates of bacterial vaginosis among women between the ages of 14 and 49 years in the United States.

METHODS: Data from the 2001–2001 and 2003–2004 National Health and Nutrition Examination Surveys were combined. Correlates of bacterial vaginosis evaluated included sociodemographic characteristics (age, race or ethnicity, education, poverty income ratio) and sexual history (age of first intercourse, number of sexual partners). Crude and adjusted odds ratios and 95% confidence intervals were estimated from logistic regression analyses.

RESULTS: Almost one third of women (29%) were positive for bacterial vaginosis. Bacterial vaginosis prevalence varied with age, race or ethnicity, education, and poverty. Black, non-Hispanic (odds ratio [OR] 3.13, 95% confidence interval [CI] 2.58–3.80) and Mexican-American (OR 1.29, 95% CI 0.99–1.69) women had higher odds of bacterial vaginosis than white, non-Hispanic women after adjustment for other sociodemographic characteristics. Douching in the past 6 months was also an important predictor of bacterial vaginosis prevalence (OR 1.93, 95% CI 1.54–2.40).

CONCLUSION: Bacterial vaginosis is a common condition among U.S. women, and the prevalence is similar to that in many treatment-seeking populations. Further studies are needed to disentangle the interactions between race or ethnicity and other sociodemographic characteristics.

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LEVEL OF EVIDENCE: III

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Sexually transmitted diseases (STDs), including infection with the human immunodeficiency virus (HIV), are important and costly public health problems in the United States. Women with bacterial vaginosis may be at increased risks for STDs, HIV, and a number of other adverse reproductive outcomes, including pelvic inflammatory disease (PID), postoperative infections, spontaneous abortion, preterm birth, and postpartum endometritis. Specifically, bacterial vaginosis has been found to be associated with the prevalence and incidence of multiple STDs, including chlamydia, gonorrhea, herpes, HIV, and trichomoniasis, and is also implicated in the development of PID.[1–8]

Bacterial vaginosis is a highly prevalent condition and the most common cause of vaginal irritation. It is a condition characterized by vaginal flora imbalance, in which normally plentiful peroxidase-producing bacteria (*Lactobacillus*) are scarce and other anaerobic bacteria abundant. The total concentration of bacteria may be 100 to 1,000 times their normal levels in women with bacterial vaginosis.[9] Many, if not most, women with bacterial vaginosis are asymptomatic. The two classic symptoms of bacterial vaginosis, discharge and odor, are reported by only a minority of affected individuals. A recent study found that, in women with bacterial vaginosis, only 25% reported odor and 42% reported discharge in the preceding 6 months.[10]

Although bacterial vaginosis is a common condition, national surveillance has been lacking. Data from the 2001–2002 National Health and Nutrition Examination Survey found bacterial vaginosis to be common among the general population (Koumans EH, Sternberg MR, McQuillan G, Bruce C, Kendrick JS, Sutton MY, et al. Prevalence of bacterial vaginosis in the United States, 2001–2002. Presented at the 2006 National STD Prevention Conference. Jacksonville, Florida, May 8–11, 2006). The objectives of this study are to 1) describe the prevalence of bacterial vaginosis among U.S. women between the ages of 14

and 49 using nationally representative data from National Health and Nutrition Examination Survey collected from 2001 to 2004, and 2) evaluate whether sociodemographic characteristics, such as race or ethnicity and age, which are correlates in clinical populations, are also associated with the prevalence of bacterial vaginosis in the general population.

MATERIALS AND METHODS

We used data from the National Health and Nutrition Examination Survey (NHANES) samples for the combined intervals 2001–2002 and 2003–2004 to estimate the prevalence of bacterial vaginosis among women in the civilian, noninstitutionalized U.S. population. The National Health and Nutrition Examination Survey, conducted by the National Center for Health Statistics at the Centers for Disease Control and Prevention, was designed to obtain nationally representative information on the health and nutritional status of the population of the United States through interviews and direct physical examinations. Methods describing this national survey have been published elsewhere.[11] This study submitted to the Washington University School of Medicine Human Subjects Committee for approval. The study was classified as exempt because it is a population-based study devoid of individual identifiers.

For these analyses, women between the ages of 14 and 49 years with bacterial vaginosis data were included. A total of 747 women were excluded from the analyses because of missing data for bacterial vaginosis. The final sample included data from 3,727 women, which when weighted represents the experience of 65,660,083 U.S. women between the ages of 14 and 49 years.

Female participants in the National Health and Nutrition Examination Survey study between the ages of 14 and 49 years were tested for bacterial vaginosis. Self-collected vaginal swabs were used for the evaluation of bacterial vaginosis. Smears were allowed to air dry before shipment to the processing and analysis laboratory at Magee-Women's Hospital (Pittsburgh, PA). The bacterial vaginosis score for Gram staining was calculated by Nugent's method.[12] Scores of 7 or higher were considered positive for bacterial vaginosis, whereas those between 4 and 6 were considered intermediate. Additional details on laboratory procedures have been published.[13,14] Prevalence was estimated for the three levels of bacterial vaginosis: positive, negative, and intermediate. For logistic regression analyses, the outcome was defined as bacterial vaginosis confirmed (positive) or not (negative and intermediate).

Self-reported sociodemographic characteristics included in these analyses included age, race or ethnicity, highest level of education received, and poverty/income ratio. Age was categorized by decade (14–19, 20–29, 30–39, and 40–49). Race or ethnicity was categorized as white, non-Hispanic; black, non-Hispanic, Mexican American; or other race/Hispanic. Three levels of education were evaluated: less than high school, completed high school (or general equivalency diploma), or more than high school. Poverty/income ratio was the ratio of the individual's family household income to the federal poverty level and was categorized as less than the federal poverty level (poverty/income ratio less than 1), at or above the federal poverty level (poverty/income ratio 1 to less than 2), or twice the federal poverty level (poverty/income ratio 2 or more).

In this analysis we examined a number of reproductive history variables. Age at first menstruation was categorized as 7–11 years, 12–14 years, 15 years or later, or unknown. Douching within the past 6 months was defined as yes, no, or unknown.

Women between the ages of 14 and 49 years also completed a sexual history questionnaire that included questions on history of sexual intercourse and lifetime and recent number of sexual partners. Because of confidentiality concerns, sexual history data for individuals between the ages of 14 and 19 years are only available at the National Center for Health Statistics Research Data Center. Therefore, all analyses including sexual history were limited to women between the ages of 20 and 49 years of age.

Categorical data were compared by using χ^2 tests. Crude and adjusted odds ratios were estimated by using logistic regression. Statistical analyses were conducted with Stata 9.2 (StataCorp, College Station, TX). Specifically, using the svyset command in Stata, we specified the individual weight, primary sampling unit, and stratum. The 2-year individual weights estimated by the National Center of Health Statistics and made available as part of the National Health and Nutrition Examination Survey data set are adjusted to the entire U.S. population based on 2000 Census information. To accommodate the joining of 2001–2002 and 2003–2004 data sets, each adjusted to the U.S. population, the weight for each individual was divided by 2 to provide a single estimate for the entire U.S. population.

RESULTS

The overall and subgroup prevalences of bacterial vaginosis are presented in Table 1. The prevalence of bacterial vaginosis in the general population of the

Table 1. Prevalence of Bacterial Vaginosis and 95% Confidence Interval Among Women 14–49 Years of Age by Sociodemographic Characteristics and Reproductive History

	Prevalence (95% Confidence Interval)			
	Positive	Negative	Intermediate	*P*
All women	29.2 (27.2–31.3)	42.8 (40.4–45.3)	28.0 (25.6–30.4)	
Sociodemographic characteristics				
Age (y)				
14–19	23.3 (20.1–26.9)	45.6 (41.5–49.7)	31.1 (28.2–34.1)	.11
20–29	31.2 (27.0–35.6)	42.2 (38.4–46.1)	26.7 (22.3–31.5)	
30–39	28.2 (25.4–31.1)	45.0 (41.7–48.4)	26.9 (23.3–30.7)	
40–49	31.3 (27.3–35.6)	40.1 (34.6–45.9)	28.6 (24.8–32.8)	
Race or ethnicity				
White, non-Hispanic	23.2 (20.8–25.8)	47.2 (44.1–50.3)	29.6 (26.2–33.2)	<.001
Black, non-Hispanic	51.6 (47.9–55.2)	27.6 (23.7–31.8)	20.9 (17.9–24.2)	
Mexican American	32.1 (27.7–36.9)	39.9 (36.0–43.8)	28.0 (23.9–32.6)	
Other race/Hispanic	36.3 (27.6–45.9)	37.1 (30.4–44.4)	26.6 (19.8–34.8)	
Education				
Less than high school	32.9 (29.6–36.4)	41.3 (37.4–45.3)	25.8 (23.1–28.7)	.01
High school/GED	33.8 (29.1–38.9)	39.0 (33.9–44.3)	27.2 (23.1–31.7)	
More than high school	25.6 (23.1–28.2)	45.1 (41.6–48.6)	29.3 (25.6–33.3)	
Poverty income ratio				
0 to 1.0	36.9 (32.3–41.7)	38.5 (33.4–43.8)	24.6 (21.7–27.8)	<.001
More than 1.0 to 2.0	34.3 (30.3–38.3)	37.4 (33.0–41.9)	28.5 (23.8–33.7)	
2.0 or more	24.1 (21.5–27.0)	46.7 (43.3–50.1)	29.2 (25.9–32.7)	
Reproductive and sexual history				
Age at menarche (y)				
7–11	29.9 (25.7–34.4)	44.1 (38.3–50.0)	26.0 (22.3–30.1)	.05
12–14	27.5 (25.2–30.0)	44.1 (41.0–47.2)	28.4 (25.7–31.3)	
15 or more	30.5 (24.4–37.5)	39.4 (32.2–47.0)	30.1 (24.4–36.6)	
Missing	39.3 (32.1–47.0)	33.4 (26.7–40.9)	27.2 (21.6–33.7)	
Douched in the past 6 months				
No	23.7 (21.7–25.8)	47.0 (44.0–50.1)	29.2 (26.7–32.0)	<.001
Yes	44.5 (41.0–48.0)	31.3 (26.3–36.8)	24.2 (19.3–30.0)	
Missing	42.1 (35.0–49.5)	32.3 (24.9–40.6)	25.7 (19.7–32.6)	
Among women aged 20–49 y				
Sexual intercourse				
Ever	29.9 (27.7–32.1)	42.3 (38.8–45.8)	27.8 (24.9–31.0)	.03
Never	14.5 (6.4–29.7)	59.3 (45.4–71.8)	26.2 (16.6–38.9)	
Missing	40.5 (33.3–48.2)	36.0 (27.4–45.6)	23.4 (16.9–31.6)	
Age of first sexual intercourse (y)				
9–14	38.2 (33.0–43.7)	33.6 (28.4–39.1)	28.3 (23.0–34.1)	.08
15–19	29.8 (26.9–32.8)	42.9 (39.3–46.7)	27.3 (24.3–30.6)	
20 or more	23.5 (17.3–31.1)	47.2 (40.3–54.1)	29.3 (20.9–39.5)	
Missing	33.2 (25.6–41.7)	42.7 (34.0–51.8)	24.2 (19.5–29.5)	
Number of male sexual partners				
Lifetime				
0	15.6 (7.7–29.0)	59.1 (46.1–70.9)	25.3 (15.9–37.8)	.01
1–2	24.7 (20.3–29.8)	46.2 (40.2–52.3)	29.1 (22.5–36.7)	
3–5	27.2 (23.1–31.8)	45.0 (39.3–50.7)	27.8 (22.5–33.8)	
6–10	34.3 (30.0–39.0)	35.7 (30.0–41.8)	30.0 (24.8–35.7)	
11 or more	25.5 (29.5–41.9)	40.9 (33.9–48.3)	23.6 (16.9–30.6)	
Missing	41.4 (34.4–48.7)	35.6 (27.9–44.1)	23.0 (16.9–30.6)	
Past 12 months				
0	36.4 (29.9–43.4)	41.2 (33.6–49.1)	22.5 (16.8–29.4)	.03
1	27.3 (25.2–29.5)	43.6 (39.8–47.4)	29.1 (25.5–33.0)	
2 or more	39.3 (33.0–45.9)	36.3 (29.1–44.2)	24.5 (18.4–31.7)	
Missing	33.4 (25.8–42.0)	42.5 (33.4–52.1)	24.2 (19.4–29.7)	

GED, general equivalency diploma.

United States was high–almost one woman in three was positive for bacterial vaginosis (29.2%). Although younger women, those between the ages of 14 and 19 years, had a somewhat lower prevalence (23.3%) of bacterial vaginosis, among the 20 years and older group the prevalence was between 28% and 31%. The prevalence of bacterial vaginosis varied significantly with race or ethnicity, education, and poverty/income ratio. Bacterial vaginosis was more common among black, non-Hispanic (51.6%) and Mexican-American (32.1%) women than among white, non-Hispanic women (23.2%). Women with more than a high school education were less likely to be positive for bacterial vaginosis than those with a high school education or less (26% versus 33–34%). Similarly, the prevalence of bacterial vaginosis was lower among those living well above the federal poverty level (24%) compared with those living at or near (34%) or below (37%) the federal poverty level.

Selected reproductive history characteristics were also associated with the prevalence of bacterial vaginosis. Although there was no noticeable trend in the relationship to bacterial vaginosis and age at menarche, women who reported douching in the past 6 months had significantly higher prevalence of bacterial vaginosis than those who did not (45% versus 24%; $P<.001$).

We examined the association between sexual history and bacterial vaginosis prevalence among women between the ages of 20 and 49 years and found that a history of sexual intercourse and number of male sexual partners were associated with bacterial vaginosis. Women who reported no history of sexual intercourse had rates of bacterial vaginosis that were half that of women who reported a history of intercourse (15% versus 30%). The prevalence of bacterial vaginosis was highest among those who reported the youngest ages of first intercourse: 38% among those who reported first intercourse between the ages of 14 and 19 compared with 30% for those aged 15–19 years and 24% for those whose first intercourse occurred at age 20 or older. Number of male sexual partners during one's lifetime and in the past year was also associated with prevalence of bacterial vaginosis. Prevalence was lowest among women with the fewest male sexual partners in their lifetimes and among those with a single male sexual partner in the past year.

Results from crude and adjusted logistic regression analyses are presented in Table 2. Although the

Table 2. Crude and Adjusted Odds Ratios and 95% Confidence Intervals for Selected Demographic Characteristics Among Women 14–49 Years of Age

	Crude	Model 1: Adjusts for Sociodemographics	Model 2: Adjusted for Sociodemographics and Douching
Age (y)			
14–19	0.67 (0.52–0.87)	0.56 (0.40–0.80)	0.65 (0.46–0.93)
20–29	–	–	–
30–39	0.87 (0.71–1.05)	0.95 (0.78–1.15)	0.91 (0.76–1.10)
40–49	1.01 (0.74–1.37)	1.17 (0.88–1.56)	1.12 (0.85–1.49)
Race or ethnicity			
White, non-Hispanic	–	–	–
Black, non-Hispanic	3.52 (2.97–4.19)	3.13 (2.58–3.80)	2.66 (2.18–3.25)
Mexican American	1.57 (1.22–2.01)	1.29 (0.99–1.69)	1.33 (1.03–1.72)
Other race/Hispanic	1.89 (1.22–2.91)	1.76 (1.13–2.75)	1.78 (1.11–2.85)
Education			
Less than high school	1.42 (1.17–1.73)	1.47 (1.13–1.92)	1.37 (1.05–1.77)
High school/GED	1.48 (1.17–1.88)	1.38 (1.08–1.75)	1.29 (0.99–1.66)
More than high school	–	–	–
Household income in relation to federal poverty below			
Below (PIR 0 to 1.0)	1.84 (1.46–2.31)	1.43 (1.17–1.74)	1.32 (1.08–1.63)
At (PIR more than 1.0 to 2.0)	1.63 (1.25–2.13)	1.34 (1.02–1.77)	1.27 (0.95–1.69)
Above (PIR 2.0 or more)	–	–	–
Douched in past 6 months			
No	–	–	–
Yes	2.57 (2.19–3.03)	–	1.93 (1.54–2.40)
Missing	2.33 (1.72–3.17)	–	1.90 (1.36–2.65)

GED, general equivalency diploma; PIR, poverty/income ratio.

odds of bacterial vaginosis were lower among women between the ages of 14 and 19 years compared with 20–29 year olds, there were no significant differences between 20–29 year olds and those at ages 30–39 years or 40–49 years. Differences observed in the crude prevalence of bacterial vaginosis by race or ethnicity, education, and poverty/income ratio persisted in the adjusted analyses. Black, non-Hispanic women had an odds of bacterial vaginosis three times that of non-Hispanic, white women after adjusting for age, education, and poverty. Similarly, the odds for Mexican-American women were slightly higher when compared with white women. The adjusted analyses confirmed associations of bacterial vaginosis with lower levels of education and living near or below the federal poverty level. These differences remained with the addition of history of douching to the model. The association with black race was attenuated somewhat (OR 2.66, 95% CI 2.18–3.25).

In the subsample of women between the ages of 20 and 49 years, we also examined the association with specific sexual characteristics after adjustment for age, race or ethnicity, education, and poverty level (data not shown). After adjustment for age, race or ethnicity, education, and poverty level, age at first intercourse and number of lifetime male sexual partners were associated with increased odds of bacterial vaginosis. Women who reported later ages of intercourse had somewhat, but not significantly, lower odds of bacterial vaginosis. Women with two to five lifetime male sexual partners did not have a higher odds of bacterial vaginosis (OR 1.03, 95% CI 0.69–1.54) than women who had one lifetime male sexual partner, but women with six or more partners did have a higher odds ratio (OR [6–10 partners] 1.47, 95% CI 1.03–2.09; OR [11 or more partners] 1.62, 95% CI 1.09–2.39). Further, having had two or more male sexual partners in the past year was associated with an increased odds of bacterial vaginosis compared with women with a single male sexual partner (OR 1.46, 95% CI 1.08–1.98).

Although preliminary, these data suggest possible interaction between race or ethnicity and other sociodemographic characteristics. Figure 1 presents the prevalence of bacterial vaginosis by age, education, and poverty/income ratio for the different strata of race or ethnicity. We sought to understand whether the relationship between age, education, and poverty were uniform across race or ethnic groups. For example, an inspection of the association with age in the different groups indicates that bacterial vaginosis prevalence increases with age among white, non-Hispanic women (P=.33, χ^2 test), is flat among Mex-

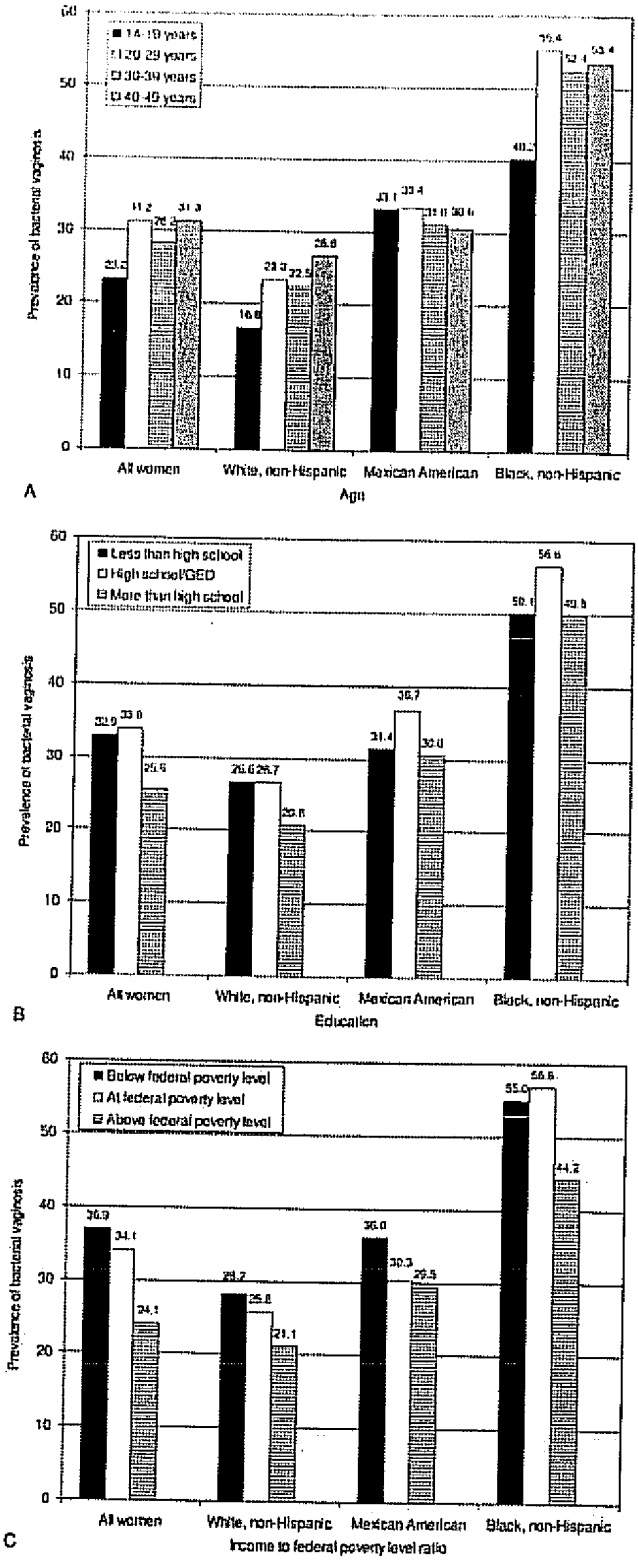

Fig. 1. Prevalence of bacterial vaginosis by age (A), education (B), and poverty/income ratio (C) stratified by race or ethnicity. GED, general equivalency diploma.
Allsworth. Prevalence of Bacterial Vaginosis. Obstet Gynecol 2007.

ican-American women (P=.70), and appears to plateau among black, non-Hispanic women from 20 years of age and older (P=.21). Similarly, when examining the association between race or ethnicity and poverty level, the prevalence of bacterial vaginosis decreases slightly, but not significantly, within increasing income for white and Mexican-American women (P=.15 and P=.16, respectively). Among black women, there was little difference in the prevalence of bacterial vaginosis among those living below (55%) or near (57%) the poverty level and a significant decrease in prevalence among those living at two times the poverty level (44%; P=.01). An additional analysis, which evaluated for interactions between race or ethnicity and sociodemographic characteristics, largely confirmed these findings. The findings were suggestive of interactions with age (P=.04), level of education (P=.07), and poverty/income ratio (P=.11) and white, non-Hispanic race or ethnicity. Further, there was a significant interaction between age and Mexican-American ethnicity. There were no significant interactions with black, non-Hispanic race or ethnicity.

DISCUSSION

Although many studies have examined the prevalence of bacterial vaginosis among different subpopulations, the National Health and Nutrition Examination Survey provides an estimate of the prevalence of bacterial vaginosis in the general U.S. population. The prevalence of bacterial vaginosis in multiple studies of pregnant women ranged from 12% to 21%,[5,15,16] was as high as 30% in people seeking health care or seeking termination of pregnancy,[17,18] and was over 50% in a population of injection drug users.[19] Yen and colleagues[3] examined women entering the military and found that 28% of the sexually experienced and 18% of non–sexually experienced women had bacterial vaginosis. Almost one third of all women tested positive for bacterial vaginosis.

Consistent with the existing literature,[18,20,21] bacterial vaginosis was more common among black and Mexican-American women. A study in women presenting at a county health center in Michigan found prevalence rates of 42% among black women, 35% among Hispanic women, and 25% among white women.[18] The prevalences for white (23%) and Hispanic (32%) women were roughly consistent with those in the general population but somewhat lower than that observed among black women (52%).

Of note, these data support the conclusion that bacterial vaginosis is not exclusively a sexually transmitted condition. Almost 15% of women who reported no history of sexual intercourse had bacterial vaginosis. This finding confirms a previous study by Yen and colleagues,[3] in which approximately 18% of sexually inexperienced women were found to be positive for bacterial vaginosis.

Previous studies have found that bacterial vaginosis prevalence increased with age. In a population of individuals seeking STD treatment, 23% of women aged 14–24 years had bacterial vaginosis compared with 33% of women aged 25 years and older.[17] Although these national data confirmed that the prevalence of bacterial vaginosis is lower among women 14 years of age, there is no evidence to support the assertion that bacterial vaginosis is associated with age among older women. This contradiction may be a consequence of clinical study designs that disproportionately enroll younger (and higher risk) women.

Socioeconomic status and poverty are also associated with the distribution of bacterial vaginosis in the population. Factors such as Medicaid status, low levels of education, absence of a telephone in the home, occupation, and employment status have all been found to be associated with higher frequency of bacterial vaginosis.[18,20,22,23]

As found in several other studies,[20,22,24,25] douching was associated with higher prevalence of bacterial vaginosis. Further many studies have found that the prevalence of douching is higher among African-American populations. In this sample 15–17% of white and Mexican-American women reported douching in the past 6 months compared with 44% of black women. The addition of douching to the adjusted model resulted in a change of the effect size of black race, but both factors remained significant predictors of bacterial vaginosis independently. Because this analysis is cross-sectional, it is not possible to ascertain whether the association with douching is causal or a result of attempts to self-treat vaginal symptoms.

Bacterial vaginosis is common among the general population of women in the United States. In fact, the prevalence of women with bacterial vaginosis in National Health and Nutrition Examination Survey was comparable with that in many treatment-seeking populations. These data confirm what has been learned about the sociodemographic distribution of bacterial vaginosis from clinical populations, namely, that race or ethnicity, education, and poverty are all associated with bacterial vaginosis prevalence. One contradiction, however, is the association with age. In contrast with previous studies, there is not an increasing prevalence with increasing age. Further, these findings indicate that the relationship between demo-

graphic characteristics and bacterial vaginosis may vary by race or ethnicity. It was not clear from subgroup analyses that associations with demographic characteristics were consistent across race or ethnicity. Bacterial vaginosis is an important predictor of adverse reproductive outcomes, and more complete understanding of the dynamics connecting these sociodemographic characteristics will allow for the creation of targeted interventions. Additional waves of National Health and Nutrition Examination Survey data will be helpful in evaluating this question in detail in the future.

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EXHIBIT N

1: J Infect Dis. 2005 Aug 1;192(3):394-8. Epub 2005 Jun 28.

Colonization of the rectum by Lactobacillus species and decreased risk of bacterial vaginosis.

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Lactobacilli colonizing the rectum may be a reservoir for vaginal lactobacilli. In a cross-sectional study of 531 females, vaginal and rectal colonization by lactobacilli were assessed by culture methods. A subset of isolates was identified to the species level by use of whole-chromosomal DNA probes. Lactobacillus crispatus (16%), L. jensenii (10%), and L. gasseri (10%) were the prevalent lactobacilli colonizing the rectums of 290 females. Only 13 (9%) of 147 females colonized by L. crispatus or L. jensenii vaginally and/or rectally had bacterial vaginosis (BV), compared with 12 (44%) of 27 females colonized by other H(2)O(2)-producing lactobacilli ($P < .001$). Cocolonization of the vagina and rectum by H(2)O(2)-producing lactobacilli was associated with the lowest prevalence of BV (5%), whereas females colonized only vaginally, only rectally, or at neither site had a successively increased risk of BV ($P < .001$). Lactobacillus species in the rectum may contribute to the maintenance of vaginal microflora.

PMID: 15995952 [PubMed - indexed for MEDLINE]

EXHIBIT O

Recurrence of *Helicobacter pylori* Infection After Eradication Therapy in Brazilian Patients With Peptic Ulcer

To the Editor:

The studies that evaluated the risk of *Helicobacter pylori* reinfection in developing countries[1,2] report a great variation in the results (1.1%–41.5% per year). In Brazil, a country with high prevalence of *H. pylori*,[3] data on the long-term reinfection are scarce.[4] We report the recurrence rate of *H. pylori* infection after successful eradication in a group of patients of low socioeconomic status with peptic ulcer followed at our gastroenterology unit.

Fifty patients (22 males; mean age, 50 ± 13 years) with peptic ulcer who participated in a previous study[5] and were successfully treated for their *H. pylori* infection with ranitidine bismuth citrate associated to clarithromycin for 2 weeks were evaluated. *H. pylori* eradication was considered to be successful when both histologic examination and urease test were negative at the time of the control endoscopy performed 3 months after the treatment of the infection. The eradication rate with this treatment was 83%. Patients were followed-up with ^{14}C-urea breath test at 6, 12, and 18 months after the termination of treatment, and with endoscopy at 12, 24, and 36 months after treatment. The probability of remaining *H. pylori*-negative over time was calculated by the Kaplan-Meier method. The mean follow-up period was 20.6 ± 8.5 months. All patients completed one year of follow-up. Thirty-four of them were followed for 18 months, 24 for 2 years, and 8 for 3 years. Recurrence of *H. pylori* infection occurred in 6 patients: 1 within 6 months of treatment, 4 during the second year, and 1 in the third year. The cumulative recurrence rate of the infection was 2%, 16.8%, and 27.2% at 1, 2, and 3 years, respectively. No ulcer recurrence was detected in this group of patients.

It is generally accepted that most early recurrences of *H.* pylori in adults are recrudescence of a suppressed infection and not true reinfection cases.[6] Considering that, it has been suggested that 3 months would be an effective and practical time interval to test for eradication.[7] This recommendation was followed in the present study. We did not perform fingerprint analysis of *H. pylori* strains, which would have confirmed that the reported recurrences were reinfection cases. However, 5 of the 6 cases of infection recurrences occurred after 1 year of follow-up and are therefore likely to be true cases of reinfection.

In summary, although the majority of patients remained free of infection after 2 years of follow-up, with no ulcer recurrence, the results of this study suggest an increasing cumulative risk of reinfection over time in our study population living in a high prevalent setting of *H. pylori*. Our results reinforce the importance of measures to improve infrastructural problems and hygienic conditions to eliminate *H. pylori* from common sources and therefore decrease the chances of reinfection in developing countries.

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Probiotics Affects Vaginal Flora in Pregnant Women, Suggesting the Possibility of Preventing Preterm Labor

To the Editor:

We read with interest the article by Reid and Devillard[1] concerning the potential effects of oral or vaginal probiotics against abnormal vaginal flora and preterm labor. Nevertheless, that article included only speculation of effects of probiotics during pregnancy. Our study is intended to clarify whether administration of probiotic lactobacilli can affect vaginal flora in pregnant women.

A randomized, controlled study enrolled 24 healthy Japanese women near full term (≥35 weeks of gestation) singleton pregnancy. The Institutional Review Board of the University of Fukui approved this study. After obtaining informed consent, we used a table of random numbers to assign patients randomly to one of two groups. For subjects allocated to the probiotics group, 120 g/day

of fermented milk containing 10^9 colony-forming units/mL of *Lactobacillus johnsonii* La1 (Nestlé Japan Ltd, Tokyo, Japan) was supplied for 2 weeks. Subjects allocated to the control group were provided placebo-fermented milk during the study period. Vaginal fluid samples were collected from each subject before and after the fermented milk administration. After incubation, various colony types were isolated and identified. In addition, lactobacilli grown on the MRS agar plates (Difco Laboratories, Detroit, MI) were counted.

In the probiotics group, pathogenic bacteria such as *Gardnerella vaginalis* and *Corynebacterium* species were detected in 4 of the 12 subjects; all of them were undetectable after the test food administration. In contrast, pathogenic bacteria were detected in 3 of the 12 subjects in the control group; all of those bacteria remained in the vagina for 2 weeks. Administration of the probiotic food significantly increased the number of vaginal lactobacilli ($P = 0.025$).

This preliminary study showed that oral administrations of probiotics can restore vaginal flora in pregnant women. Our results strongly support Reid's theory.[1,2] Bacterial vaginosis is a risk factor for preterm labor, especially in high-risk pregnant women.[3] Further studies are needed, but probiotics use is of potential benefit to pregnant women. Probiotics are an effective method to prevent or treat bacterial vaginosis and related disease during pregnancy.

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Supported in part by a 2001 Grant-in-Aid for scientific research from the Applied Science Board for Lactic Acid Bacteria.

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Necrotizing Fasciitis Secondary to Intraabdominal Infection in Crohn's Disease

To the Editor:

Necrotizing fasciitis is a life-threatening infection with mortality rates of 10% to 20%; cases arising secondary to intraabdominal infection were unusual, and most such reported cases prove fatal.[1–4] Crohn's disease is an inflammatory bowel disease and often associated with infection, but cases resulting in necrotizing fasciitis are also rare.[5,6] We report a case of a necrotizing fasciitis with Crohn's disease that required surgical therapy including laparotomy.

A 26-year-old woman was referred from an emergency unit for sacroiliac pain with high fever. She had had no history and symptoms of bowel disease before. She had swelling of the abdominal wall spreading to the sacroiliac area with erythema with a white blood cell count of 36.9×10^9/L, indicating severe inflammation. Skin incision and drainage were immediately performed, and *Escherichia coli* was isolated from the pus and necrotizing skin. Abdominal CT scan revealed that the rectus abdominal muscle was ruptured and an abscess was found between the abdominal muscle and the posterior layer of abdominal sheath, but there were no fistulae and rupture on peritoneum.

Two weeks later, surgery removing all the dead tissue was carried out. The whole cavity, which showed continuity from the paraumbilical area to the right iliac crest toward the back, was left open widely and a free skin grafting was performed to resurface the whole wound. Intraoperative examination showed no fistulae lead to the intraabdominal cavity. All the wounds were heal within 1 month. However, 6 weeks later, she developed a small fistula at the paraumbilical area that passed to the intraabdominal cavity, so she underwent laparotomy. The fistula passed into the abdominal cavity and communicated with the ileum that adhered to the abdominal inner wall. The diseased bowel was resected, and the patient made a full recovery. Histologic examination showed longitudinal ulcer, pseudopolyposis, and skip lesion, which indicated Crohn's disease.

Necrotizing fasciitis is an aggressive and life-threatening infection but rarely originated from intraabdominal processes. In our case, the perforation of inflammatory ileum on 2 days before admission would have been an onset, but clear signs of peritonitis were not identified. Furthermore, severe subcutaneous soft tissue inflammation hindered discovery during the clinical examination. Gerber et al indicated, in their 2 cases of necrotizing fasciitis secondary to ruptured appendicitis and diverticulitis, that the abdominal examination was surprisingly benign with no rigidity or tenderness.[1]

Progressive necrotizing fasciitis in Crohn's disease is quite unusual and has been reported only by two investigators.[5,7] In both cases, patients were diagnosed with Crohn's disease beforehand and followed up. In contrast to those cases, our case showed uncommon course in which the patient had presented no bowel symptoms before, and proved to be Crohn's disease after laparotomy.

It is concluded that necrotizing fasciitis secondary to intraabdominal processes is unusual but can deal the patient a fatal blow, so the early recognition and debridement of the involved tissue are essential and represent the only treatment of these aggressive infections.

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EXHIBIT P

1: Microbes Infect. 2006 May;8(6):1450-4. Epub 2006 Mar 29.

Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14: randomized, double-blind, placebo controlled trial.

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This study enrolled 125 premenopausal women diagnosed with bacterial vaginosis (BV) by presence of vaginal irritation, discharge and 'fishy' odor, and Nugent criteria and detection of sialidase enzyme. The subjects were treated with oral metronidazole (500 mg) twice daily from days 1 to 7, and randomized to receive oral Lactobacillus rhamnosus GR-1 (1 x 10 (9)) and Lactobacillus reuteri RC-14 (1 x 10(9)) or placebo twice daily from days 1 to 30. Primary outcome was cure of BV as determined by normal Nugent score, negative sialidase test and no symptoms or signs of BV at day 30. A total of 106 subjects returned for 30-day follow-up, of which 88% were cured in the antibiotic/probiotic group compared to 40% in the antibiotic/placebo group ($p<0.001$). Of the remaining subjects, 30% subjects in the placebo group and none in the probiotic group had BV, while 30% in the placebo and 12% in the probiotic group fell into the intermediate category based upon Nugent score, sialidase result and clinical findings. High counts of Lactobacillus sp. (>10(5) CFU/ml) were recovered from the vagina of 96% probiotic-treated subjects compared to 53% controls at day 30. In summary, this study showed efficacious use of lactobacilli and antibiotic in the eradication of BV in black African women.

PMID: 16697231 [PubMed - indexed for MEDLINE]

system, gastrointestinal tract, skin, vagina, and other areas of the body. Antibiotics will not work for colds, flu, or...

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EXHIBIT Q

1: Microbes Infect. 2006 Oct;8(12-13):2772-6. Epub 2006 Sep 11.

Clinical study comparing probiotic Lactobacillus GR-1 and RC-14 with metronidazole vaginal gel to treat symptomatic bacterial vaginosis.

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Bacterial vaginosis (BV) is particularly common in black women, and in Nigeria it is often caused by Mycoplasma, as well as Atopobium, Prevotella and Gardnerella sp. Antimicrobial metronidazole oral therapy is poorly effective in eradicating the condition and restoring the Lactobacillus microbiota in the vagina. In this study, 40 women diagnosed with BV by discharge, fishy odor, sialidase positive test and Nugent Gram stain scoring, were randomized to receive either two dried capsules containing Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 each night for 5 days, or 0.75% metronidazole gel, applied vaginally twice a day (in the morning and evening). Follow-up at day 6, 15 and 30 showed cure of BV in significantly more probiotic treated subjects (16, 17 and 18/20, respectively) compared to metronidazole treatment (9, 9 and 11/20: P=0.016 at day 6, P=0.002 at day 15 and P=0.056 at day 30). This is the first report of an effective (90%) cure of BV using probiotic lactobacilli. Given the correlation between BV and HIV, and the high risk of the latter in Nigeria, intravaginal use of lactobacilli could provide women with a self-use therapy, similar to over-the-counter anti-yeast medication, for treatment of urogenital infections.

PMID: 17045832 [PubMed - indexed for MEDLINE]

areas of the body. Antibiotics will not work for colds, flu, or...

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EXHIBIT R

1: Can J Microbiol. 2005 Sep;51(9):777-81.

Vaginal microbial diversity among postmenopausal women with and without hormone replacement therapy.

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Urogenital infections in postmenopausal women remain problematic. The use of estrogen replacement therapy has been shown to lower these infection rates, corresponding to increasing colonization by Lactobacillus species. Despite the gut's 500 microbial species and the proximity of the anus to the vagina, only a relatively few microbial strains appear to be able to colonize the urogenital area. In the present study, the sparsity of microbes in the vagina was confirmed by denaturing gradient gel electrophoresis analysis of swabs taken at time zero and monthly for 3 months from 40 postmenopausal subjects receiving Premarin (conjugated equine estrogen in combination with progesterone) hormone replacement therapy (HRT) and 20 who were not on HRT. Lactobacilli were recovered from the vagina of 95% or more women in both groups, but in the HRT group, Lactobacillus were more often the dominant and only colonizers and significantly fewer bacteria with pathogenic potential were found. The incidence of bacterial vaginosis was significantly lower in the HRT group than in the non-HRT-treated women (5.6% versus 31%). The use of HRTs has recently come under criticism. The ability of drugs such as Premarin to help recover the lactobacilli vaginal microbiota appears to be at least one benefit of HRT use. In women not using HRTs, use of probiotics may be the only way to restore a nonpathogen-dominated flora.

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and sweating) in women who are experiencing menopause ('change of life', the end of monthly menstrual periods). Some brands of estroge...

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